Catalytic Activity of Crystalline Aspartate Aminotransferase

INAUGURAL-DISSERTATION
zur Erlangung der Philosophischen Doktorwürde

vorgelegt der
Philosophischen Fakultät II
der
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von
HEINZ KIRSTEN
aus Hasle bei Burgdorf/BE

Begutachtet von Herrn Prof. Dr. Ph. Christen

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Dedicated to my parents
and to my wife Sylvia

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- Structure and Dynamics of the Active Site of Mitochondrial Aspartate Aminotransferase. Eichele, G., Ford, G.C., Jansonius, J.N., Halonbrenner, R., Kirsten, H., Hausner, U., Wilson, K.J., and Christen, P. (1979), 11th Annual Meeting of the Swiss Societies for Experimental Biology (USGEB), Genf, 1979, Experientia 35, 933; 11th International Congress of Biochemistry, Toronto, 1979, Abstracts, p. 195.

- The Three-Dimensional Structure of Mitochondrial Aspartate Aminotransferase at 2.8 Å Resolution: Crystallographic and Spectrophotometric Studies of its Active Site. Jansonius, J.N., Eichele, G., Ford, G.C., Christen, P., Hausner, U., Kirsten, H., Wilson, K.J., and Halonbrenner, R., Pyridoxal Symposium, Toronto, 1979, Abstracts, p. 7.

- Mitochondrial Aspartate Aminotransferase: Functional Groups at the Active Site, Syncatalytic Changes in Conformation, in vitro Synthesis of a Putative Precursor. Christen, P., Gehring, H., Jaussi, R., Kirsten, H., Pfister, K., Sandmeier, E., Sonderegger, P., Jansonius, J.N., Eichele, G., and Ford, G.C., Pyridoxal Symposium, Toronto, 1979, Abstracts, p. 8.

- Catalytic Properties of Crystalline Mitochondrial Aspartate Aminotransferase, Lattice-Induced Functional Asymmetry of the Two Subunits. Kirsten, H., Gehring, H., and Christen, P., Abstracts of the 13th Annual Meeting of the Swiss Societies for Experimental Biology (USGEB), Lausanne, 1981, Experientia 37, 627; 14th Meeting of the Federation of European Biochemical Societies, Edinburgh, 1981, Biochemical Society Transactions, Abstracts, p. 323.

- Conformational Changes during Catalysis by Mitochondrial Aspartate Aminotransferase. Jansonius, J.N., Eichele, G., Ford, G.C., Kirsch, J.F., Picot, D., Thaller, C., Vincent, M.G., Christen, P., Gehring, H., and Kirsten, H., Meeting of the American Society of Biological Chemists, 1981, Fed. Proc. 40, 1757.

The following publications are in preparation:

- Catalytic Activity of Non-Crosslinked Microcrystals of Aspartate Aminotransferase in Polyethylene Glycol. Kirsten, H., and Christen, P.

- Crystalline Aspartate Aminotransferase: Lattice-Induced Functional Asymmetry of the Two Subunits. Kirsten, H., Gehring, H., and Christen, P.

- Kinetic Properties of Microcrystalline Aspartate Aminotransferase. Christen, P., and Kirsten, H., in Transaminases, Christen, P., and Metzler, D.E. (eds.), Wiley, New York.

Abbreviations

A_{max}	Maximum absorption
A_{min}	Minimum absorption
AspAT	Aspartate aminotransferase (mitochondrial isoenzyme)
PEG	Polyethylene glycol ($M_r \sim 6000$)
PEG solution	30% (w/v) polyethylene glycol, 50 mM sodium phosphate, pH 7.5. For the linear dichroism studies of single crystals 20% (w/v) PEG was used in the same buffer. Other concentrations of the two components used are indicated in the text.

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SUMMARY

Microcrystals of mitochondrial aspartate aminotransferase were grown in the presence of 15% (w/v) polyethylene glycol. Fractionation of the suspension yielded microcrystals of approximately uniform size (average dimensions 22 x 5 x 0.8 μm). The crystals were triclinic and of the same habit as those used for high resolution X-ray crystallographic analysis (Ford, G.C., Eichele, G., and Jansonius, J.N. (1980) Proc. Natl. Acad. Sci. USA 77, 2559-2563). The enzymic activity of suspensions of microcrystals was measured photometrically in 30% (w/v) polyethylene glycol. Under these conditions, the solubility of the enzyme is very low (saturation concentration 0.15 μg/ml) dispensing with crosslinking of the crystals. The molar activity of the crystalline enzyme is one order of magnitude lower than that of the enzyme in solution. Kinetic tests ruled out that diffusion of the substrates into the crystals was rate-limiting. The observed decrease in the catalytic efficiency can be attributed exclusively to crystal packing effects, because polyethylene glycol ($M_r \sim 6000$) cannot penetrate the liquid channels of the crystals. The catalytic competence of the crystalline enzyme warrants the relevance of the model of the spatial structure for the elucidation of details of the enzymic mechanism of action.

In triclinic crystals the molecular dyad of the enzyme dimer does not coincide with any of the crystallographic axes. The two subunits thus have dissimilar lattice contacts. Determination of the Michaelis-Menten parameters showed that the asymmetric packing of the enzyme dimer into the crystal lattice not only decreases its activity but also induces a functional nonequivalence of the two subunits that behave identically in solution (K_m' for aspartate 0.3 mM, and for 2-oxoglutarate 1.1 mM). The crystalline enzyme possesses a high affinity

subunit (K_m' for aspartate 0.5 mM, and for 2-oxoglutarate 1.2 mM) and a low affinity subunit (K_m' 5.5 mM and 14.5 mM, respectively); the molar activity is 3.2% and 15% of that of the enzyme in solution, respectively.

The notion of a lattice-induced functional nonequivalence of the two active sites of the enzyme dimer is corroborated by the possibility of selective mechanism-based elimination of either type of active sites. To a suspension of microcrystals in the pyridoxamine form oxalacetate was added in substoichiometric amounts such that only a fraction of all subunits was converted to the pyridoxal form. At the same time, excess sodium borohydride was added. Under these conditions, the subunits undergoing transamination faster will be inactivated preferentially by reduction of the Schiff base of the newly formed pyridoxal form. The preferential inactivation of the subunits that contribute more to the measured activity of the crystalline enzyme manifests itself in a greater decrease in the activity of the suspension of microcrystals than in the activity measured after dissolution of the crystals. As expected, in the converse experiment starting with the crystal suspension in the pyridoxal form the other type of subunit is inactivated preferentially.

X-ray crystallographic examination of the apoenzyme complex with the coenzyme-substrate analog N-(5'-phosphopyridoxyl)-L-aspartate revealed a marked conformational change in only one of the two subunits, the S* subunit (Eichele, G. (1980) Ph. D. Thesis, University of Basel). Kinetic tests based on the functional nonequivalence of the two subunits of the crystalline enzyme dimer led to a tentative identification of the low and high affinity subunit with the S and S* subunit, respectively. In view of the identical conformation of the two subunits as shown by X-ray crystallographic analysis, the functional asymmetry of the crystalline enzyme is very likely due to a difference in the constraints exerted by lattice forces on the conformational adaptability of the two subunits.

The decreased activity of crystalline aspartate aminotransferase supports the importance of the already known ligand-induced and syn-catalytic conformational changes in bringing about the most efficient energy profile of the enzymic transamination reaction. Linear dichroism studies with single crystals of aspartate aminotransferase showed that the coenzyme ring undergoes a rotatory movement during the formation of coenzyme-substrate intermediates. This observation is complementary to similar X-ray crystallographic findings and fits the concept of a dynamic mechanism of action of this enzyme.

ZUSAMMENFASSUNG

Aspartat-Aminotransferase wurde in Gegenwart von Polyäthylenglykol 6000 kristallisiert. Durch Fraktionierung der Mikrokristallsuspension erzielte man eine annähernd einheitliche Grösse der Kristalle (durchschnittliche Dimensionen 22 x 5 x 0.8 µm). Diese Kristalle waren triklin und hatten den gleichen Habitus wie diejenigen, welche für die hochauflösende Röntgenstrukturanalyse verwendet worden waren (Ford, G.C., Eichele, G., und Jansonius, J.N. (1980) Proc. Natl. Acad. Sci. USA 77, 2559-2563). Die enzymatische Aktivität von Mikrokristallsuspensionen wurde photometrisch in 30% (Gew./Vol.) Polyäthylenglykol bestimmt. Unter diesen Bedingungen ist die Löslichkeit des Enzyms sehr niedrig (Sättigungskonzentration 0.15 µg/ml). Ein Quervernetzen der Kristalle war somit nicht notwendig. Die molare Aktivität kristalliner Aspartat-Aminotransferase ist um eine Zehnerpotenz niedriger als diejenige des Enzyms in Lösung. Eine Beschränkung der Geschwindigkeit der enzymatischen Reaktion durch das Eindiffundieren der Substrate in die Kristalle wurde durch kinetische Tests ausgeschlossen. Da Polyäthylenglykol ($M_r \sim 6000$) nicht in die Flüssigkeitskanäle der Kristalle eindringen kann, ist die beobachtete Abnahme der katalytischen Wirksamkeit auf die Packung des Enzymmoleküls in das Kristallgitter zurückzuführen. Die katalytische Kompetenz des kristallinen Enzyms leistet Gewähr, dass das Modell der Raumstruktur als Grundlage zur Aufklärung von Einzelheiten des enzymatischen Wirkungsmechanismus der Aspartat-Aminotransferase dienen kann.

Die beiden Untereinheiten der Aspartat-Aminotransferase sind bezüglich einer zweizähligen molekularen Achse symmetrisch. In triklinen Kristallen fällt diese Achse mit keiner der drei Kristallachsen zusammen. Dadurch ergeben sich für die beiden Untereinheiten unter-

schiedliche Gitterkontakte mit ihren Nachbarmolekülen. Die Bestimmung der Michaelis-Menten Parameter zeigte, dass die asymmetrische Packung des Enzymdimers im Kristall nicht nur die katalytische Aktivität herabsetzt, sondern auch eine funktionelle Ungleichheit der beiden Untereinheiten herbeiführt. In Lösung reagieren die Untereinheiten des Dimers unabhängig voneinander und sind funktionell gleichwertig (K_m' 0.3 mM für Aspartat und 1.1 mM für 2-Oxoglutarat). Das kristalline Enzym hingegen besitzt eine hochaffine Untereinheit (K_m' 0.5 mM für Aspartat und 1.2 mM für 2-Oxoglutarat) und eine niederaffine Untereinheit (K_m' 5.5 mM und 14.5 mM). Die molare Aktivität der Untereinheiten ist 3.2% bzw. 15% derjenigen des Enzyms in Lösung.

Die Feststellung einer gitterinduzierten funktionellen Ungleichheit der beiden Untereinheiten des Dimers wird durch die Möglichkeit, selektiv die hochaffine oder niederaffine aktive Stelle zu inaktivieren, bestätigt. Zu Mikrokristallen in der Pyridoxaminform gab man Oxalacetat in substöchiometrischer Menge, so dass nur ein Teil der Untereinheiten in die Pyridoxalform zurückgeführt wurde. Gleichzeitig gab man einen Ueberschuss an Natriumborhydrid zu, um die neugebildete interne Schiff'sche Base der schneller transaminierenden Untereinheit zu reduzieren. Die Aktivität des kristallinen Enzyms nahm dabei stärker ab als die nach Auflösen der Kristalle gemessene Aktivität. Dieser Unterschied ist zurückzuführen auf die bevorzugte Inaktivierung derjenigen Untereinheiten, die einen höheren Beitrag zur gemessenen Aktivität der kristallinen Aspartat-Aminotransferase liefern. Führt man das umgekehrte Experiment durch, d.h. gibt man die Aminosäure Cysteinsulfinat zur Pyridoxalform der Mikrokristalle, so werden erwartungsgemäss die anderen Untereinheiten bevorzugt inaktiviert.

Die röntgenkristallographische Untersuchung des Komplexes des Apoenzyms mit dem Coenzym-Substrat-Analogen N-(5'-Phosphopyridoxyl)-L-aspartat zeigten deutliche konformationelle Aenderungen des Enzymproteins nur in einer der beiden Untereinheiten, der sogenannten

S*-Untereinheit (Eichele, G. (1980) Dissertation, Universität Basel). Kinetische Untersuchungen erlaubten, die S*-Untereinheit als die hochaffine Untereinheit zu identifizieren. Die röntgenanalytische Untersuchung hat gezeigt, dass die beiden Untereinheiten im Kristall dieselbe Konformation aufweisen. Die funktionelle Ungleichheit der Untereinheiten scheint dadurch zustande zu kommen, dass die Gitterkräfte deren konformationelle Anpassungsfähigkeit in unterschiedlichem Ausmass beschränken.

Die erniedrigte Aktivität des kristallinen Enzyms weist hin auf die Bedeutung der bereits bekannten liganden-induzierten und synkatalytischen Konformationsänderungen des Enzyms zur Erreicnung eines optimalen Energieprofils der Transaminierungsreaktion. Messungen des Linear-dichroismus von Einzelkristallen der Aspartat-Aminotransferase zeigten, dass der Coenzymring während der Bildung von Coenzym-Substrat-Zwischenverbindungen eine Drehbewegung ausführt. Diese Beobachtung ergänzt ähnliche röntgenkristallographische Befunde und stimmt mit der Vorstellung eines dynamischen Wirkungsmechanismus dieses Enzyms überein.

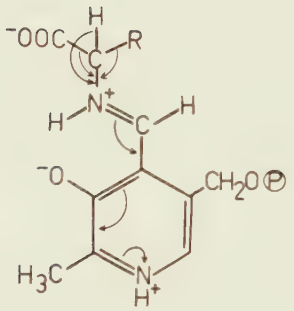
I GENERAL INTRODUCTION

1. General Aspects of Nonenzymic Pyridoxal Catalysis and Catalysis by Aminotransferases

Pyridoxal-5'-phosphate is the biochemically active derivative of vitamin B_6 which serves as prosthetic group of many enzymes in the metabolism of amino acids. A general mechanism of pyridoxal catalysis is based on studies of nonenzymic reactions of amino acids with the cofactor (Braunstein and Shemyakin,1953; Metzler et al., 1954). A common feature of pyridoxal-5'-phosphate-dependent reactions is the initial formation of a Schiff base between the aldehyde group of the cofactor and the amino group of the substrate. An important rate-enhancing factor is the formation of a conjugated system of double bonds extending from the reaction site to the strongly electrophilic pyridinium nitrogen (Scheme I). As a consequence, the displacement of a pair of electrons from one of the bonds of the 2-carbon atom to its substituents is facilitated. The manifold reactions of the coenzyme substrate adduct form the basis for the observed variety of pyridoxal-5'-phosphate-dependent reactions. In enzymic pyridoxal-5'-phosphate-dependent catalysis, the enzyme of course determines not only the reaction pathway taken by the coenzyme substrate adduct but also the rate of the reaction. A comprehensive compilation of transamination reactions has been assembled by Braunstein (1973).

The initial step of the enzymic reaction is a transaldimination rather than de novo formation of the Schiff base with the amino acid substrate. Optical and chemical studies have shown that pyridoxal-5'-phosphate is covalently bound via a Schiff base linkage to the 6-amino group of a lysine residue of the enzyme. A hypothetical

Scheme I



- Displacement of the proton from C-2 of the amino acid moiety in transamination reactions, racemizations, 3-eliminations (e.g. serine dehydratase), and 3-replacement (e.g. tryptophan synthase)
- Displacement of carbon dioxide by amino acid decarboxylases
- Displacement of the side chain (R^+), e.g. serine transhydroxymethylase.

mechanism of the transamination reaction as catalyzed by aminotransferases involving 2-amino acids and 2-oxo acids is represented in Scheme II (courtesy of Dr. E. Sandmeier). It has been verified both in nonenzymic model reactions and in studies of enzymic transamination. The main steps in transamination reactions are: transaldimination, tautomerization and hydrolysis (for a review, see Braunstein, 1973).

Hypothetical Mechanism of Aminotransferases (double displacement reaction):

Transaldimination

- Pyridoxal-5'-phosphate, covalently bound to the 6-amino group of Lys 258 by a Schiff base linkage, i.e. *internal aldimine* (I).
- Nucleophilic addition of the amino acid substrate to the imino bond, formation of a *tetrahedral intermediate* (II).
- Release of Lys 258 yielding the *external aldimine* (III).

Tautomerization

- Elimination of the proton at the 2-carbon from the external aldimine (III) yields a *semiquinonoid intermediate* (IV) of which three resonance structures are depicted.
- Reprotonation at the 4'-carbon atom results in formation of a *ketimine* (V).

The tautomerization, the rate-limiting step of the enzymic transamination reaction, is base-catalyzed.

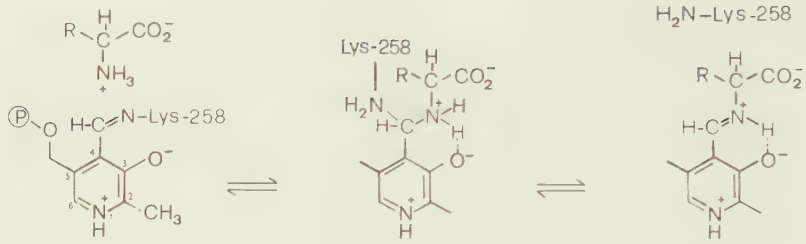
Hydrolysis

- The imino double bond of the ketimine (V) is hydrolyzed yielding the *pyridoxamine form* (VI) of the enzyme and the 2-oxo acid product.

In the second half-reaction of transamination the pyridoxamine form (VI) reacts with another 2-oxo acid regenerating the pyridoxal form by the same steps in reverse succession and forming the corresponding new amino acid.

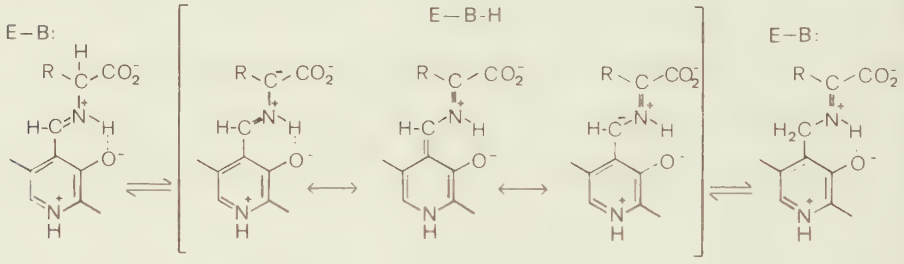
Scheme II

Transaldimination



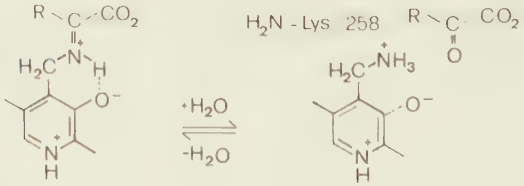
I II III

Tautomerization



III IV V

Hydrolysis



V VI

2. Molecular and Functional Properties of Aspartate Aminotransferase

The functional and structural properties of AspATs from various sources have been described aiming at a comprehensive model of the catalytic site and a dynamic mechanism of action (for a review, see Braunstein, 1973). In animal cells two isoenzymes of AspAT, *i.e.* the cytosolic and the mitochondrial isoenzyme, are found. Both isoenzymes are synthesized in the cytosol, the mitochondrial enzyme as a precursor with higher molecular weight (Sonderegger et al., 1980; Jaussi et al., 1982). The translocation of the mitochondrial isoenzyme from the cytosol into the mitochondria is under investigation (Sonderegger et al., 1982; Jaussi et al., 1982). To date, amino acid sequences of seven AspATs from different species are known (Ovchinnikov et al., 1973; Doonan et al., 1975; Shlyapnikov et al., 1979; Barra et al., 1979; Kagamiyama et al., 1980; Huynh et al., 1980; Graf-Hausner et al., manuscript in preparation). The comparison of these primary structures has shown that the cytosolic and the mitochondrial isoenzymes are homologous proteins.

Successfull crystallization of AspAT and X-ray crystallographic analyses led to models of the spatial structure at high resolution of three AspATs: the cytosolic enzyme from pig (Arnone et al., 1977, 1982) and from chicken (Borisov et al., 1980), and the mitochondrial isoenzyme from chicken (Ford et al., 1980). AspAT is a dimer of identical subunits. The two subunits of the crystalline enzyme are related by a molecular dyad except those regions which are involved in lattice contacts (Eichele et al., 1979). Each subunit is characterized by two domains of different size, the larger of the two binds the coenzyme. Each active site is situated in a pocket generated by the two subunits, *i.e.* composed from amino acid residues of both subunits (Ford et al., 1980). The access of the substrate to the active site is partially blocked by the frontally bridging NH_2-terminal segment

of the polypeptide chain which extends from the small domain of one subunit to the coenzyme binding domain of the other subunit (Eichele et al., 1979; Ford et al., 1980). This bridging segment of the polypeptide seems to be both of functional and structural significance (Sandmeier and Christen, 1980).

AspAT catalyzes the following reaction:

L-glutamate + oxalacetate $\rightleftarrows$ 2-oxoglutarate + L-aspartate

During catalysis the coenzyme shuttles between the pyridoxal form and the pyridoxamine form. In solution, the two active sites of the enzyme dimer undergo the transamination reaction independently as shown by manifold criteria such as the determination of the activity of hybrid dimers with one active and one inactive subunit (Schlegel and Christen, 1974, 1978; Lee and Churchich, 1975; Boettcher and Martinez-Carrion, 1975, 1976) and the determination of the active site occupancy pattern at transamination equilibrium (Schlegel et al., 1977).

The successive steps in transamination involve conformational adaptations of the enzyme. Ligand-induced and syncatalytic conformational changes of the protein moiety were first observed by modifying cysteine residue 390 of cytosolic AspAT and cysteine residue 166 of mitochondrial AspAT. The reactivity of both Cys 390 and Cys 166 toward modifying reagents is altered in different enzyme-substrate intermediates (Birchmeier et al., 1973; Gehring and Christen, 1978). Changes in the conformation during catalysis were also detected by infrared spectroscopy measuring the peptide hydrogen-deuterium exchange (Pfister et al., 1978).

A movement of the coenzyme ring during the transamination reaction was early proposed by Ivanov and Karpeisky (1969) in their dynamic mechanism of action. High resolution X-ray crystallographic analyses (Ford

et al., 1980) and linear dichroism measurements (Metzler et al., 1978; Makarov et al., 1981) have confirmed such a movement of the cofactor ring.

3. Functional Properties of Enzymes in the Crystalline State

Structural information on enzymes in solution may be obtained by various spectroscopic methods. However, the most detailed pictures of the spatial structures of enzymes have been realized by high resolution X-ray crystallographic analyses of large single crystals (for a review, see Blundell, 1976). The difference Fourier technique provides a sensitive monitor of conformational changes involving the enzyme protein and the coenzyme moiety. In studies on the structure-function relationship of an enzyme including experimental data obtained both in the crystalline state and in solution the question is raised to what degree the enzyme's structure in the crystal resembles that in solution. Crystallization may select particular conformations or induce certain conformational changes such that the enzyme cannot assume the conformations necessary for catalytic efficiency. The effect of the crystal lattice on the catalytic activity of the enzyme depends on the particular set of molecular lattice contacts established. For example, different crystal types of hexokinase showed different catalytic properties, one crystal form even being completely inactive (Anderson et al., 1974). Similar observations were made with tetragonal crystals of glycogen phosphorylase b. The enzyme molecule appears to be constrained so that it cannot respond to the binding of the allosteric effector AMP (Johnson et al., 1979).

An estimate of the catalytic efficiency of the crystalline enzyme is essential for assessing the relevance of the model of the spatial structure as determined by X-ray crystallography for the elucidation of the details of the mechanism of action. Any conclusion about the

structural basis of the catalytic activity should rely on a comprehensive study of the structure and the function of the enzyme in the same physical state. In the present study, the catalytic properties of AspAT were determined under the same conditions as used for X-ray crystallographic analysis (see *Section II*).

Crystalline proteins are well ordered samples suited for studies of conformational changes by measurements of linear dichroism. Linear dichroism refers to the orientation-dependent absorption of plane-polarized light of oriented matter (for a review, see Hofrichter and Eaton, 1976). The dichroic behavior of the coenzyme chromophore of crystalline AspAT was studied in different transamination intermediates in order to assess its syncatalytic reorientation as detected previously by X-ray crystallographic analysis (Ford et al., 1980). A qualitative interpretation of the present linear dichroism data obtained from triclinic crystals is given in *Section III*.

II CATALYTIC PROPERTIES OF CRYSTALLINE ASPARTATE AMINOTRANSFERASE, LATTICE-INDUCED FUNCTIONAL NONEQUIVALENCE OF THE TWO SUBUNITS

1. Introduction

The purpose of this study is to compare the functional properties of crystalline AspAT with those of the enzyme in solution. The differences found will have to be considered when a detailed catalytic mechanism is deduced from the model of the enzyme's spatial structure as it is provided by X-ray crystallographic analysis (Ford et al., 1980) in conjunction with the determination of the amino acid sequence (Graf-Hausner et al., manuscript in preparation). The crystallographic analysis at a resolution of 2.8 Å was performed with triclinic crystals containing one enzyme dimer per unit cell. The crystals were grown in PEG. Microspectrophotometry of single crystals of AspAT has shown that the absorption spectra of the coenzyme chromophore in the different functional states of the enzyme are almost identical with those of the enzyme in solution and that all active sites of the enzyme dimers in the crystal are capable to undergo the two half-reactions of transamination (Eichele et al., 1978; Metzler et al., 1978; Mozzarelli et al., 1979). On soaking in 20% (w/v) PEG solutions containing the appropriate substrate, the enzyme crystal underwent transamination without having impaired its crystalline order (Eichele et al., 1978). However, the catalytic activity of the crystalline enzyme could not be determined by microspectrophotometry because of the diffusional rate limitation in the relatively large-sized crystals used for these measurements. By measuring the enzymic activity in suspension of microcrystals diffusional rate limitation may be precluded. To date, only few crystallized enzymes have been examined in this way (for reviews, see Rupley, 1969; Makinen and Fink, 1977). More recent

studies were reported on lactate dehydrogenase (Bayne and Ottesen, 1976), carboxypeptidase A (Spilburg et al., 1977), carboxypeptidase B (Alter et al., 1977) and subtilisin (Tüchsen and Ottesen, 1977). These previous investigations were performed with microcrystals that were either covalently crosslinked or kept insoluble in highly concentrated salt solutions.

In the present study, PEG at 30% (w/v) concentration reduced the solubility of the enzyme sufficiently for dispensing with crosslinking the microcrystals. Thus, any interference of uncontrolled modifications of the crystalline enzyme with its catalytic action was avoided. In contrast to salt ions or organic solvents of other crystallization media, PEG of high molecular weight ($M_r \sim 6000$ in this study) cannot penetrate the interior of the crystals because of its random coil behavior giving it a large Stokes radius (McPherson, 1976; Ingham, 1977). The liquid channels surrounding the enzyme molecules in the crystal (Fig. 1, courtesy of Dr. G. Eichele) and giving the substrates access to the active sites are thus filled with buffer solution only. Any difference in the catalytic efficiency between the crystalline enzyme and the enzyme in solution must be due to crystal packing effects and not to direct effects of the crystallization medium. A further advantage of non-crosslinked crystals is the convenient determination of the active site concentration by measuring the activity of the enzyme after dissolution of the crystals.

In AspAT, the two subunits of the enzyme in solution have proven functionally equivalent by manifold criteria (Schlegel and Christen, 1974, 1978; Lee and Churchich, 1975; Boettcher and Martinez-Carrion, 1975, 1976; Schlegel et al., 1977). In the crystals of the unliganded

Fig. 1. Assembly of AspAT dimers in the crystal lattice. The electron density was sliced perpendicular to the molecular twofold axis (○). The molecular boundary of the central dimer is outlined.

enzyme no structural asymmetry of the dimer is detectable by X-ray crystallographic analysis at 2.8 Å resolution (Ford et al., 1980). In triclinic crystals as used in the studies mentioned above, the molecular dyad does not coincide with any of the crystallographic axes. Thus, the possibility exists that dissimilar lattice contacts induce a functional nonequivalence of the two subunits. Indeed, examination of the interactions of crystalline AspAT with productive substrates has revealed that the packing of the enzyme dimer into the crystal lattice not only decreases its activity but also results in a functional nonequivalence of the two subunits. To date, AspAT provides the only example of an oligomeric enzyme where such a lattice-induced functional asymmetry of subunits has been detected by noncrystallographic means.

2. Experimental Procedure

Materials. Mitochondrial AspAT was isolated from chicken heart as described previously (Gehring et al., 1977a). All other chemicals used were of the highest available purity. Polyethylene glycol 6000 for synthetic purpose was purchased from Merck. DL-Vinylglycine was a gift from Dr. R.R. Rando. N-(5'-Phosphopyridoxyl)-L-aspartate was synthesized according to Severin et al. (1969), and Khomutov et al. (1971). The pH value of buffered solutions was adjusted after the addition of PEG. PEG, 30% (w/v), shifts the pH value of 50 mM sodium phosphate, pH 7.5, by 0.25 units toward the alkaline (Eichele et al., 1978; Atha and Ingham, 1981). Crosslinked microcrystals were prepared by treatment with 25 mM glutaraldehyde in 30% (w/v) PEG, 50 mM sodium phosphate, pH 7.5 for 5 s at room temperature. The reaction was stopped by diluting the incubation medium 10 times with buffer containing no PEG. Subsequently, the suspension was centrifuged for 15 min at 1500 x g. The sediment was resuspended in the same buffer. The

activity measured in the supernatant of crosslinked microcrystals was < 0.1% of the total activity. The enzymic activity of crosslinked microcrystals markedly depended on the extent of exposure to glutaraldehyde. In crystals crosslinked as described above it was 6% of that of the enzyme in solution regardless whether it was measured in the presence or absence of 30% (w/v) PEG.

Assay for AspAT activity in solution. The photometric assay coupled with malate dehydrogenase (EC 1.1.1.37 from pig heart, purchased from Boehringer) was performed according to Karmen (1955) in 50 mM aspartate, 50 mM 2-oxoglutarate, 50 mM sodium phosphate, 0.4 mM NADH, 1.6 U/ml malate dehydrogenase, pH 7.5 at 25°C. The reaction was started by addition of 5 to 50 µl AspAT (final concentration 0.01 to 0.1 U/ml) to 2.990 ml of the assay solution. In an alternative assay not depending on malate dehydrogenase, the reaction was followed by the absorption of oxalacetate generated (ε_{280} = 499 $M^{-1}cm^{-1}$; Nisonoff et al., 1952) in the same assay solution without NADH and malate dehydrogenase (total volume 1.5 ml).

Assay for alanine aminotransferase activity in solution. The assay solution was 400 mM alanine, 6 mM 2-oxoglutarate, 50 mM sodium phosphate, 0.6 mM NADH, 14 U/ml lactate dehydrogenase (EC 1.1.1.27 from rabbit muscle, purchased from Boehringer), pH 7.5, at 25°C in a 0.5 cm cuvet. The reaction was started by addition of 10 µl AspAT (∿ 10 U AspAT activity/ml assay) to 1.045 ml of the assay solution. The apparent activity was corrected for NADH consumed in the reduction of 2-oxoglutarate catalyzed by lactate dehydrogenase (Holbrook et al., 1975).

Protein concentrations in suspension of microcrystals were determined after dissolution of the crystals in 50 mM sodium phosphate, pH 7.5, either spectrophotometrically (ε_{280} = 1.4 x 10^5 $M^{-1}cm^{-1}$ for the enzyme dimer) or by measuring the enzymic activity.

3. Results and Discussion

3.1. Preparation and Characterization of Suspensions of Microcrystals

Crystallization. To a solution of AspAT (5 to 8 mg/ml) in 50 mM sodium phosphate, pH 7.5, 0.2 mM pyridoxal-5'-phosphate was added. After 20 min at room temperature, and addition of 2 mM 2-oxoglutaric acid, the solution was passed over a Sephadex G-25 column that was eluted with 50 mM sodium phosphate, pH 7.5. The enzyme solution was concentrated to 10 mg/ml by ultrafiltration (PM 10 membrane from Amicon); the specific activity was 200 U/mg.

Crystallization was performed in a polystrol test tube (60 x 9 mm) equipped with a magnetic stirring bar and kept at 25°C in a water bath. To the concentrated enzyme solution (300 µl) an equal volume of 30% (w/v) PEG, 50 mM sodium phosphate, pH 7.5 (PEG solution) was added dropwise under continuous stirring. The enzyme solution became turbid during the addition of the last drops of the PEG solution. Within 2 min, the first crystals were discernible under the light microscope (magnification 70 x). After 5 min, the crystals had reached an average length of 22 µm. The crystals were transferred into PEG solution by centrifuging twice at 1500 x g in a swing-out rotor for 1 min at room temperature and suspending the sediment in 300 µl PEG solution. In order to inhibit microbial growth 0.2 mg/ml sodium azide was added. ApoAspAT, prepared as described elsewhere (Schlegel and Christen, 1974), was crystallized by exactly the same procedure.

Fractionation of microcrystals. Five suspensions (300 µl each) obtained as above were combined and subjected to the following protocol: (1) The tube (60 x 9 mm) containing the crystal suspension is sealed with Parafilm and rotated end-over-end with 2 rpm at room temperature in the dark for 5 h. Apparently, this procedure results in a reorganization of part of the crystals eliminating a high fraction of non-

sedimentable material after dilution of the suspension in the presence of substrates (see *"Saturation Concentration"*). (2) The tube is kept in vertical position at 4°C in the dark until a loose sediment has been formed after 1 to 3 days. (3) The supernatant containing very small crystals and some amorphous material is removed with a pipet. The sediment is suspended in the same tube with 700 µl PEG solution. The tube is kept in vertical position for 1 h at room temperature and then centrifuged at 200 x g for ∿ 10 s. (4) The supernatant is transferred into another tube of the same dimensions and again centrifuged at 1500 x g for 90 s. The sediment is suspended in PEG solution to give an enzyme concentration of ∿ 5 mg/ml. After a new addition of sodium azide the crystal suspensions are stored in the dark at 4°C.

Crystallization of thicker microcrystals. The first steps were the same as those described under *"Crystallization"*. However, after formation of the microcrystals 150 µl PEG solution were added anew. Subsequently, 300 µl enzyme solution (10 mg/ml) were added dropwise (within 2 min) under continuous slow stirring. The magnetic rod was then removed and the tube was subjected to the slow end-over-end rotation described above for maximally 45 min. Subsequently the PEG concentration in this suspension (13% w/v) was increased to 17% by adding 3 x 100 µl PEG solution in intervals of 15 min. In between the tube was continuously rotated. The crystals were transferred into PEG solution by two subsequent centrifugations as described under *"Crystallization"*. After rotating the tube for 5 h, it was held vertically at room temperature for 30 min until a sediment began to form. After centrifugation at 200 x g for 15 s the sediment was suspended in 1 ml PEG solution and the centrifugation was repeated. The sediment was again suspended in PEG solution and azide was added.

Scanning electron microscopy. A sample of the crystal suspension (10 µl) was added to 50 µl PEG solution containing 1.8% (w/v) glutaral-

dehyde and kept at room temperature for 30 min. The crosslinked crystals were suspended in 0.5 ml water and applied onto a Nuclepore filter (from Nuclepore Corporation, pore diameter 1 µm) that had been positioned on a sintered glass funnel using slightly reduced pressure (53.3 kPa). The crystals were dehydrated by a series of ethanol/water mixtures with increasing ethanol content: 50, 70, 80, 90, 96% (v/v). In each solution the crystals were kept for 10 min, the last step was repeated. The crystals were dried in a sputtering device (Balzers-Union) at 6.7 Pa for 10 min and sputtered with gold ions under argon (13.4 Pa). Inspection of the crystals with a scanning electron microscope (Cambridge S4) showed them to be of fairly uniform size (Fig. 2).

3.2. Saturation Concentration of Aspartate Aminotransferase in Solutions of Polyethylene Glycol

The enzymic activity of non-crosslinked microcrystals can be obtained reliably by subtracting the activity in the centrifugal supernatant of the assay solution from the total activity measured provided that the contribution of the dissolved enzyme to the measured activity is not too high. The following experiments served to assess the saturation concentration of the enzyme in the PEG solution under assay conditions. Concentrated stock suspensions of crystals were used in order to transfer a minimum amount of dissolved enzyme into the substrate containing PEG solution (assay PEG solution). In timed intervals, the concentration of enzyme in the supernatant of the assay PEG solution was determined (Fig. 3). The constant level of enzyme in the supernatant reached after 5 to 7 h is independent of the total amount of microcrystals added and is assumed to correspond to the saturation concentration (0.15 µg/ml assay PEG solution). In the first 2 to 3 h the assay PEG solution seems to be supersaturated. The initial maximum concentration of nonsedimentable enzyme increases with

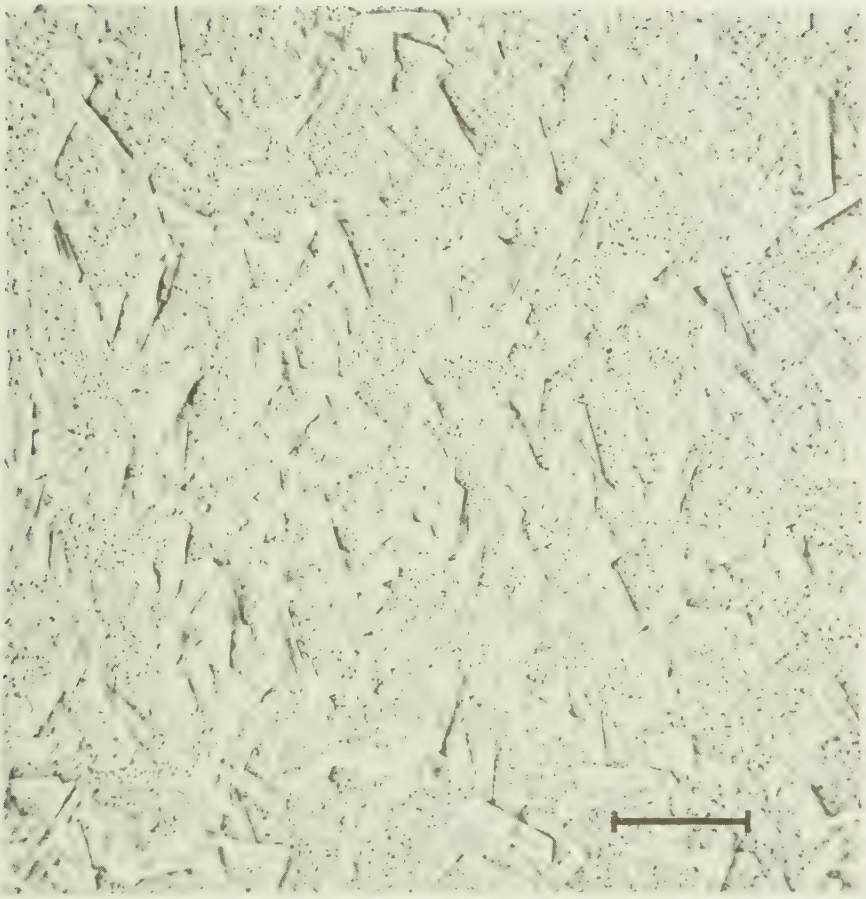


Fig. 2A. Scanning electron micrograph of microcrystals of mitochondrial (AspAT. The bar indicates 50 µm, pore diameter of Nuclepore filter 1 µm (courtesy of Dr. U. Jauch and D. Metzger, Institut für Pflanzenbiologie, University of Zurich). The microcrystals are of the same habit as those used for X-ray crystallographic analysis (Ford et al., 1980). Their average dimensions are 22 x 5 x 0.8 µm. The thickness of the crystals was measured by tilting the object table by 90° (the value of 0.8 µm is the mean of 30 crystals). A comparison of the length of very large crystals (250 x 30 x 30 µm) as measured in the native state under the light microscope with the length of dehydrated crosslinked crystals as measured with the scanning electron microscope did not show significant differences.

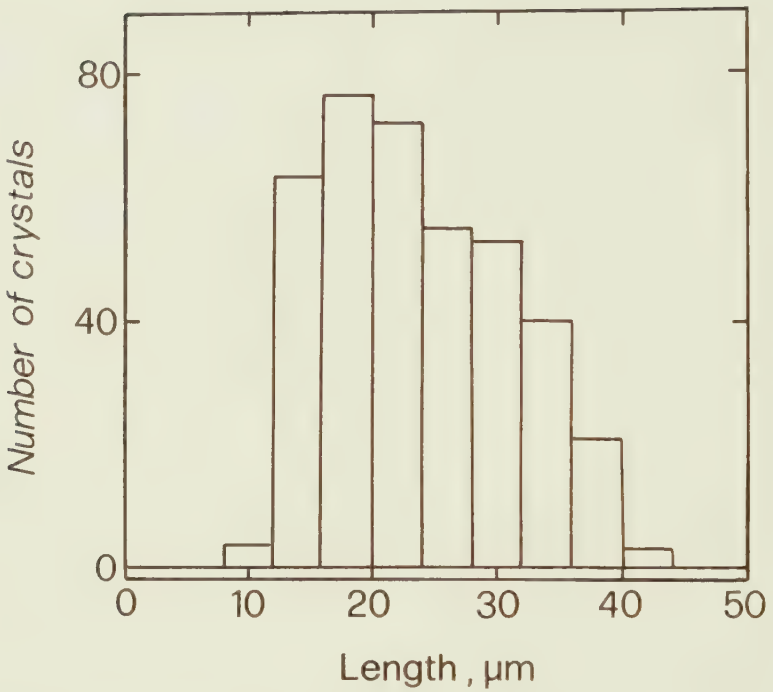


Fig. 2B. Histogram of length of crystals as measured on scanning electron micrographs. Average length 22 µm. The crystals were from 3 different crystallization batches.

increasing total amounts of microcrystals added. End-over-end rotation of the stock suspension significantly decreases the fraction of nonsedimentable enzyme that is found after dilution of the stock suspension in the assay PEG solution (inset Fig. 3). The fraction of enzyme in the supernatant exceeding the saturation concentration probably exists in the form of nonsedimentable aggregates. Apparently, these aggregates are metastable, the component molecules slowly adding to microcrystals. The aggregates are too small to be detected by scanning electron microscopy in the residue after filtration of the

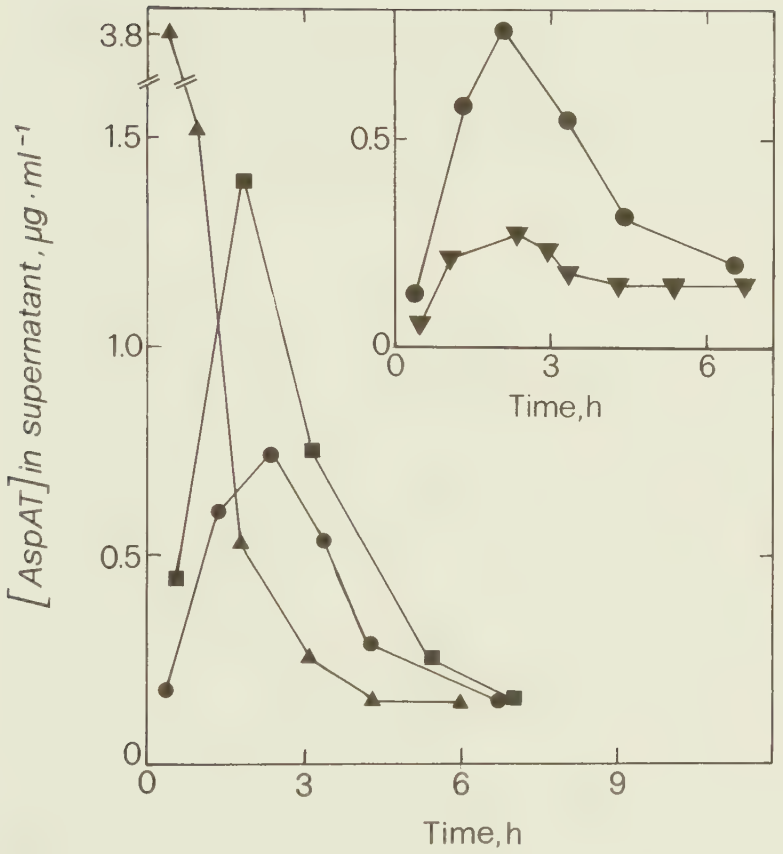


Fig. 3. Determination of the saturation concentration of AspAT by adding crystalline enzyme to assay PEG solution. The concentration of enzyme in the supernatant is given as a function of time at different total concentrations of microcrystalline AspAT: 1.5 mg/ml (▲), 0.07 mg/ml (■), 0.03 mg/ml (●). Stock suspensions of freshly crystallized AspAT that had not been subjected to the end-over-end rotation procedure were concentrated by centrifugation and transferred (concentrations used 4.5 and 43.5 mg/ml) to 1.45 ml assay PEG solution containing 150 mM aspartate and 6 mM 2-oxoglutarate in a polystrol test tube. The tube was sealed with Parafilm and continuously rotated end-over-end for preventing sedimentation of the crystals. In timed intervals, 100 µl samples were centrifuged in a swing-out rotor at 54,000 x g for 20 min at 25 ± 2°C (for details,

see *"Catalytic Activity of Crystalline AspAT"*). The enzyme concentration in the supernatant was determined by its activity in an assay without PEG. The specific activity as measured in an assay without PEG was unchanged at the end of the experiments. The inset shows the effect of preceding end-over-end rotation of the suspension of microcrystals (1.5 mg/ml, ▲), after rotating for 5 h the suspension was diluted with assay PEG solution to 0.03 mg/ml (▼). The experiment with nonrotated crystals at the same concentration (●) is replotted.

supernatant (Nuclepore filter, pore diameter 1 μm). The transient overshoot in the fraction of nonsedimentable enzyme observed after centrifugation of the stock suspension and transferring a sample of it into the assay PEG solution may reflect physical damage of part of the crystals resulting in formation of such metastable nonsedimentable aggregates. End-over-end rotation of the stock suspension reduces the overshoot, most likely by inducing a reorganization of the most fragile crystals. Foltmann (1959) in describing solubility studies with crystalline chymosine reported that the rate of dissolution was diminished in the case of crystals that had been used in a previous experiment. The overshoot concentration corresponds to maximally 2% of the total protein. Inspection of the microcrystals by scanning electron microscopy before and after the end-over-end rotation did not reveal any difference. In the present study, the suspension of newly crystallized AspAT to be used for experimentation was routinely subjected to the end-over-end rotation procedure (see *"Crystallization"*).

The saturation concentration of AspAT in the assay PEG solution was also estimated by starting from dissolved enzyme rather than from a suspension of microcrystals. Different amounts of dissolved AspAT were added to an assay PEG solution. The ensuing enzymic activity was compared with that in the supernatant obtained by high-speed centrifugation (Fig. 4). The concentration of enzyme in the supernatant was determined by measuring the enzymic activity in an assay without PEG. The activity of AspAT in assay PEG solution is 40 to

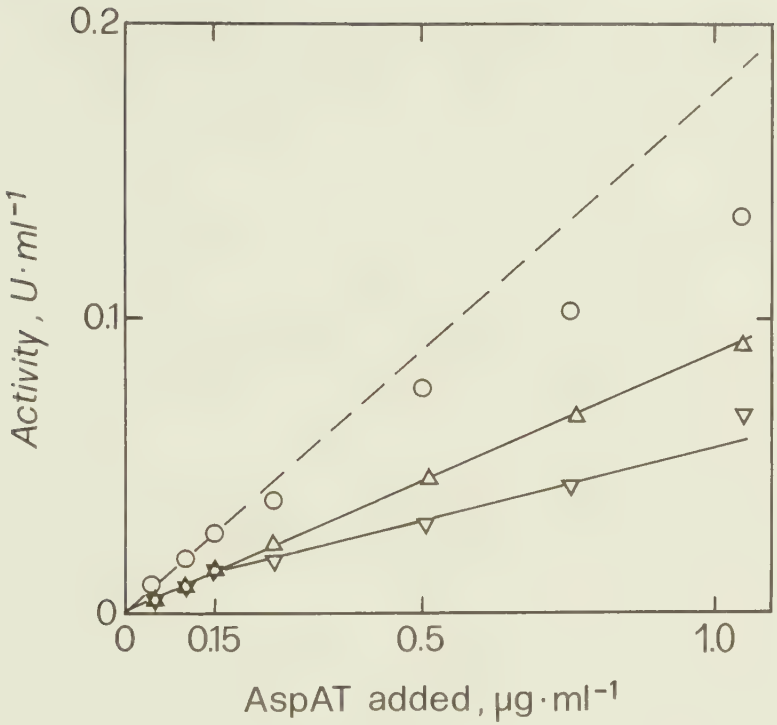


Fig. 4. Determination of the saturation concentration by adding dissolved AspAT to assay PEG solution. A constant volume (10 µl) of an appropriately prediluted stock solution of AspAT was added to 1.47 ml assay PEG solution to give the indicated final concentrations. The assay PEG solution contained 30% (w/v) PEG, 150 mM aspartate, 6 mM 2-oxoglutarate, 0.4 mM NADH, 2.5 U/ml malate dehydrogenase (activity measured in PEG solution). The enzymic activity was determined immediately after the addition of the enzyme before centrifugation (△) and in the supernatant after centrifugation (▽) at 54,000 x g, 20 min , 25 ± 2°C (for details on the assay and the centrifugation, see *"Catalytic Activity of Crystalline AspAT"*). As a measure of the enzyme concentration in the supernatant, the enzymic activity was determined in an assay without PEG (○) at the same substrate concentrations.

50% of that in the absence of PEG. The activity increases as a linear function of enzyme concentration in the total range tested indicating that the effect of PEG is independent from the state of aggregation of the enzyme. At enzyme concentrations < 0.15 µg/ml no activity is lost on centrifugation. Above this limit, increasing amounts of sedimentable activity are removed by centrifugation. Thus, both the preceding experiment and that starting with dissolved enzyme indicate a saturation concentration of 0.15 µg AspAT/ml assay PEG solution. The sedimentable material generated above the saturation limit is not crystalline. Microscopic inspection of the sediment shows an amorphous precipitate. If the deficit in enzymic activity of the supernatant were due to centrifugal removal of crystalline enzyme, 4 to 5 times more enzymic activity as measured in the assay without PEG would be lost from the supernatant, because the enzymic activity of the crystalline enzyme is only ∿ 10% of that of the enzyme in solution without PEG. The amount of nonsedimentable material increases with increasing amounts of enzyme added even above the saturation limit. This material must represent small metastable aggregates of the same kind that account for the transient overshoot of nonsedimentable material in the experiment of Fig. 3. Over the whole concentration range tested, the enzymic activity of the supernatant measured in the presence of PEG is 40 to 50 % of that measured in its absence, again indicating that PEG decreases the enzymic activity of AspAT regardless of its state of aggregation, viz. molecularly dispersed, nonsedimentable metastable aggregates, or sedimentable amorphous material. The inhibition must be due to a direct interaction between PEG and AspAT. The effect is small, it nevertheless points out that the inertness of PEG toward proteins as reported in other cases (Amiconi et al., 1977; Atha and Ingham, 1981) may not apply generally.

A further experiment confirming the above estimate of the saturation concentration started also with dissolved AspAT. The enzyme was added to assay PEG solutions of varying PEG concentrations. The activity of

the enzyme was determined as a function of the concentration of PEG and the concentration of the enzyme (Fig. 5). At all enzyme concentrations tested, the activity decreases slightly with increasing PEG concentration. At a certain PEG concentration the decrease becomes

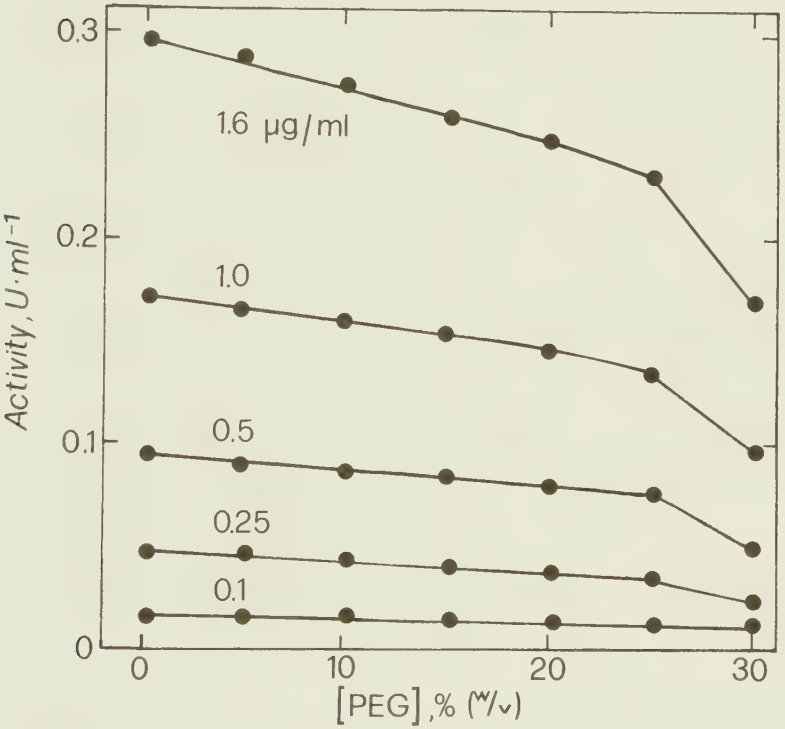


Fig. 5. Determination of the saturation concentration by adding dissolved AspAT to assay PEG solutions containing increasing concentrations of PEG. A constant volume (10 µl) of appropriately prediluted stock solutions of AspAT was added to 1.47 ml assay PEG solution to give the indicated final concentrations from 0.1 to 1.6 µg/ml. The concentration of the other components in the assay PEG solution were 150 mM aspartate, 6 mM 2-oxoglutarate, 0.4 mM NADH, and 2.5 U/ml malate dehydrogenase.

steeper, the PEG concentration at which the bend occurs being the lower the higher the enzyme concentration. At protein concentrations > 0.1 µg/ml the bend occurs between 25 and 30 % (w/v) PEG corresponding with the estimate of the saturation concentration of the protein in 30% (w/v) PEG as determined in the preceding experiments (see Figures 3 and 4).

For measuring the enzymic activity of suspensions of microcrystals, it is not possible to correct for the contribution of noncrystalline enzyme to the measured activity by simply subtracting a constant value corresponding to the activity of enzyme at saturation concentration. Due to the overshoot phenomenon several hours are required to establish the saturation concentration in the supernatant, *i.e.* the equilibrium between crystalline and molecularly dispersed enzyme (Fig. 3). In the activity assays taking only a couple of minutes after addition of the suspension of microcrystals, the fraction of noncrystalline enzyme is not constant but increases on the ascending limb of the overshoot peak (see Fig. 3). Because the extent of the overshoot depends on the concentration of the crystalline enzyme added and varies somewhat for the different batches of crystal suspensions used, the activity measured in each assay was corrected by the activity measured in the corresponding supernatant (5 to 20% of total, see below).

3.3. Catalytic Activity of Crystalline Aspartate Aminotransferase

The enzymic activity in suspensions of microcrystals was assayed in 0.5 cm cuvets (other dimensions 1.0 x 4.4 cm), thermostated at 25°C. On one side, a helical plexiglass stirrer (diameter 0.4 cm, pitch 1.6 cm) was dipped into the cuvet; through the other side the light beam passed (slit width 4 mm in both the Eppendorf photometer and the Hitachi Perkin Elmer spectrophotometer, type 340). The stirring

rate was 800 rpm. The assays were performed in PEG solution containing the substrates aspartate and 2-oxoglutarate at the indicated concentrations. To 1.40 ml of this solution 20 µl NADH (final concentration 0.4 mM) and 50 µl malate dehydrogenase (final concentration 2.5 U/ml [a]) were added. The reaction was started by addition of 5 to 10 µl of a suspension of microcrystals (giving 0.02 to 0.15 U/ml assay), with a capillary pipet (uniform diameter 1 mm). The reaction progress curves were linear until all NADH had been consumed (at low enzyme concentration for 20 min). Routinely, the reactions were followed for 3 to 5 min. Subsequently, the total assay suspension was centrifuged in a 1.5 ml polypropylene tube with a swing-out rotor (SW 27) in an ultracentrifuge (Beckman L5-65) at 54,000 x g for 20 min. Centrifugation was started when the pressure had reached 0.07 Pa. Setting the temperature control of the centrifuge to 26°C, the temperature of the sample was kept at 25 ± 2°C as measured with a thermocouple after the centrifugation. The supernatant (1.3 ml) was transferred with a Pasteur pipet into a polystrol test tube and mixed with newly added NADH and malate dehydrogenase (final concentration 0.4 mM and 2.5 U/ml, respectively) on a vibratory mixer. The new addition of malate dehydrogenase was necessary because about 40% were lost in the sediment. Subsequently, the solution was transferred into a cuvet and the enzymic activity was measured as described above. The enzymic activity of the microcrystals was obtained by subtracting the activity of the supernatant from the total activity measured in the crystal suspension. Depending on the amount of crystals in the assay, the correction was 5 to 20% of the total activity. The enzymic activity of suspensions of

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[a] The activity of malate dehydrogenase was measured under stirring in 30% (w/v) polyethylene glycol, 0.5 mM oxalacetate, 0.2 mM NADH, 0.1 M sodium phosphate, pH 7.5, 25°C (Bergmeyer, 1974). The measured activity was 30% of the activity (1200 U/ml) measured in the absence of PEG. The assays were linear with time and enzyme concentration (2 to 20 µg/ml assay solution).

thin microcrystals (average thickness 0.8 μm, see Fig. 2) was independent of the stirring rate from 700 to 3000 rpm. The activity of thicker crystals (average thickness 6.5 μm) was also independent of the stirring rate from 700 to 1600 rpm (at higher stirring rates the thicker crystals were cracked). Thus, the measured activity is not influenced by Nernst diffusion layer effects at the solution-crystal interface (Ginzburg and Katchalsky, 1963). In an alternative assay (same as described above for AspAT in solution), the reaction was followed by the absorption of oxalacetate generated (ε_{280} = 920 M^{-1} cm^{-1}). The higher extinction coefficient of oxalacetate in the PEG solution probably is due to a shift in the keto-enol equilibrium of oxalacetate.

The molar activity of microcrystalline AspAT measured at 50 mM aspartate and 100 mM 2-oxoglutarate is 10 % of that of the enzyme in solution (Table I). Both methods for following the enzymic reaction in the crystal suspension, *i.e.* the coupled assay with malate dehydrogenase and direct photometric determination of oxalacetate formed, gave the same results.

3.4. Estimation of Diffusional Rate Limitation in Microcrystals

Concentration gradients of the substrates in the interior of the crystals arise when their diffusion in the liquid channels is slow in comparison to the reaction rate. Such concentration gradients will result in a lowered reaction rate in the center of the crystal, particularly at substrate concentrations near or below the K_m values of the enzyme. A quantitative theory elaborated by Katchalski et al. (1971) and by Laidler and Bunting (1980) describes the kinetic properties of enzymes immobilized in artificial matrices. An important part of these studies were the specification of experiments for assessing diffusional rate limitation in immobilized enzymes. In the work of Spilburg et al.

TABLE I. Catalytic Activity of Crystalline AspAT per Subunit

	Aspartate	Alanine
Crystalline enzyme	900 min^{-1} [a]	0.3 min^{-1} [c]
	990 min^{-1} [b]	0.1 min^{-1} [d]
Enzyme in solution [e]	9000 min^{-1}	0.5 min^{-1}

[a] The activity of thin microcrystals (average thickness 0.8 μm) was determined with the coupled test with malate dehydrogenase. The concentrations of aspartate and 2-oxoglutarate were 50 mM and 100 mM, respectively. The measured activity was a linear function of enzyme concentration in the range from 1 to 55 μg/ml.

[b] Activity was determined by direct photometric measurement of oxalacetate formed. The substrate concentrations were the same as in a. The measured activity was a linear function of enzyme concentration in the range from 1 to 33 μg/ml.

[c] The same activity was measured in suspensions of thin and thick microcrystals (average thickness 0.8 and 6.5 μm).

[d] Determined with single crystals (thickness ∿ 10 to 30 μm) with a microspectrophotometer.

[e] Determined as described under *"Experimental Procedure"*.

(1977) the theory was applied to carboxypeptidase A immobilized by crystallization. The concentration of the active sites in the interior of the crystals was varied with an irreversible inhibitor, and the ensuing effect on the catalytic activity was determined. If there is no rate limitation by diffusion of reactants in the crystals, the activity will be a linear function of the concentration of active sites in the crystals. If the activity is limited by diffusion it will become a function of the square root of the active site concentration in the crystals.

For the estimation of the diffusional rate limitation of microcrystalline AspAT, the active site concentration, *i.e.* the concentration of active subunits in the microcrystals, was decreased with the enzyme-activated inhibitor vinylglycine. The irreversible inhibition is due to the enzyme-catalyzed transformation of vinylglycine to the 2,3-unsaturated enamine and subsequent nucleophilic attack of the 6-amino group of the active site lysine residue (Gehring et al., 1977b). The reaction of vinylglycine with the crystalline enzyme was followed by measuring the decrease in enzymic activity (Fig. 6). The slow rate of reaction ($t_{1/2}$ = 30 min) warrants a uniform distribution of the inactivated subunits throughout the interior of the crystals. Consistent with this assumption, the same relative decrease in enzymic activity of the modified crystals is measured in the crystal suspension and after dissolution of the crystals. The measured activity was taken to represent the concentration of the active subunits.

The enzymic activity of microcrystals that had been inactivated with vinylglycine to different degrees was measured at a substrate concentration below the K_m' values. In the case of thin crystals (average thickness 0.8 µm) the activity was a linear function of the concentration of active subunits in the crystals indicating the absence of diffusional rate limitation (Fig. 7).

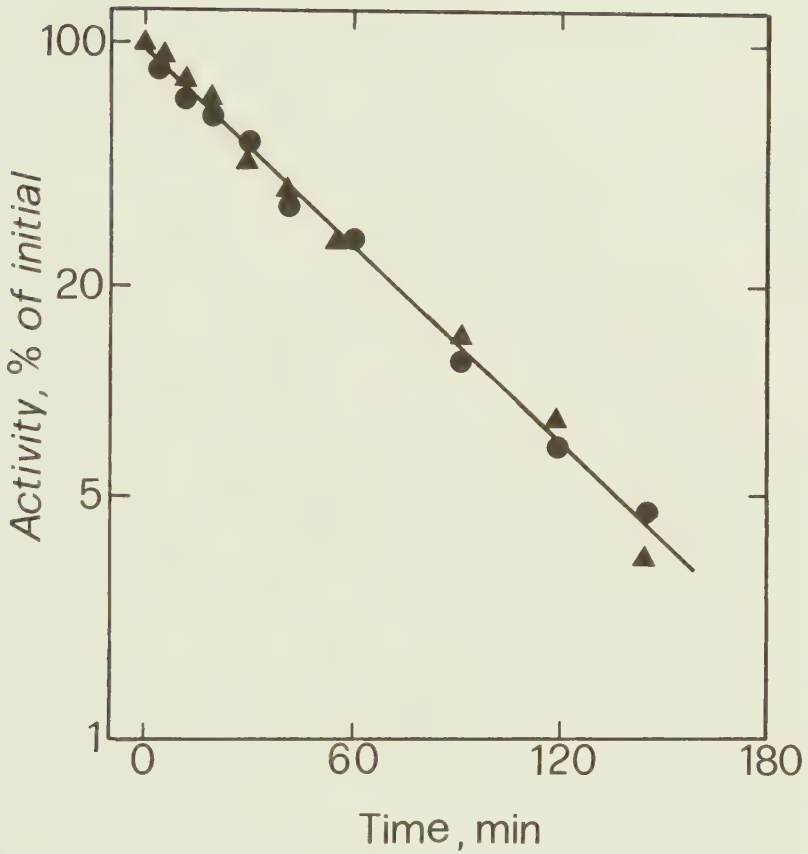


Fig. 6. Inactivation of microcrystalline AspAT by the enzyme-activated inhibitor vinylglycine. Relative activity of microcrystals (▲) and after dissolution of the crystals in assay without PEG (●). DL-Vinylglycine (19 mM) was added to a suspension of microcrystals (pyridoxal form, subunit concentration 0.16 mM, average thickness 0.8 μm) in PEG solution containing 3.5 mM 2-oxoglutarate.

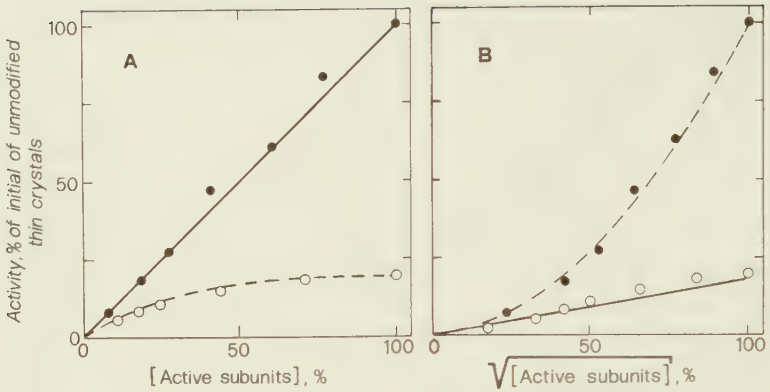


Fig. 7. Estimation of diffusional rate limitation of thin and thick microcrystals. The concentration of the active subunits was varied by the enzyme-activated inhibitor vinylglycine (see Fig. 6). The activities of the microcrystals were measured at a substrate concentration below K_m', *i.e.* with 0.2 mM aspartate (K_m' 0.5 mM) and 6 mM 2-oxoglutarate. A similar plot was obtained when the enzymic activities were measured at 0.2 mM 2-oxoglutarate (K_m' 1.2 mM) and 150 mM aspartate. Enzymic activities are expressed in percent of the initial activity of unmodified thin crystals. The concentrations of active subunits are related to the initial value of dissolved thin and thick unmodified microcrystals, respectively, measuring the activities in an assay without PEG after dissolution of the crystals.

A, shows the dependence of the activity of thin (●, average thickness 0.8 μm) and thick (○, average thickness 6.5 μm) microcrystals on the concentration of active subunits. B, shows the same enzymic activities plotted as a function of the square root of the concentration of active subunits.

3.5. pH/Activity Profile

PEG is excluded from the interior of the crystals (McPherson, 1976; Ingham, 1977). A comparison of the pH/activity profile of the crystalline enzyme with that of the enzyme in solution shows that the profile of the crystalline enzyme is shifted by ∿ 0.5 pH unit toward lower pH values while retaining its shape (Fig. 8). In the total pH

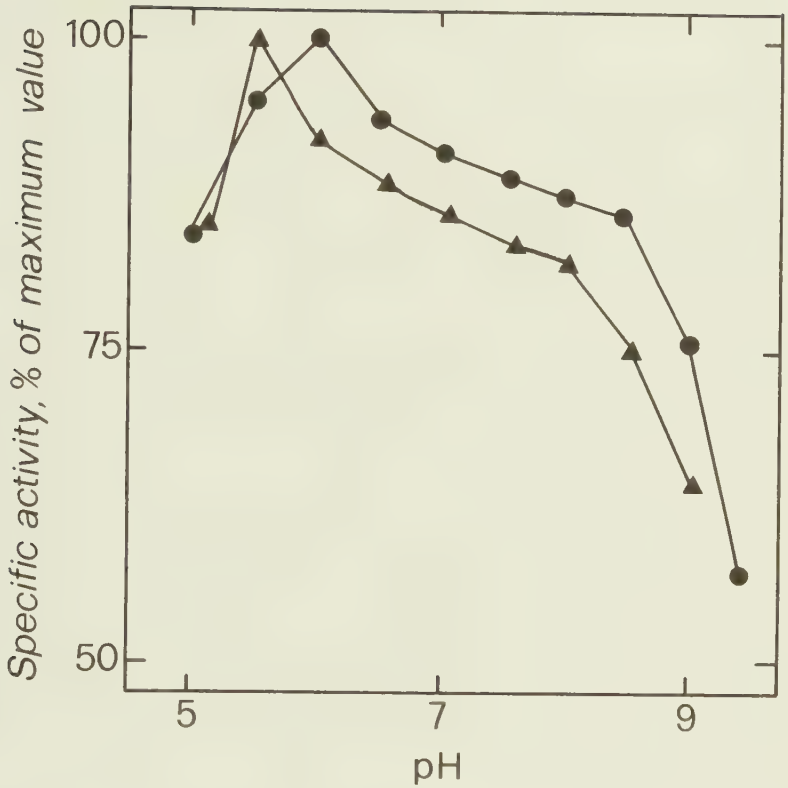


Fig. 8. Effect of crystallization on the pH/activity profile of AspAT. The activities of crystalline AspAT (▲) and of enzyme in solution (●) were measured in 50 mM sodium acetate, 50 mM sodium phosphate in the presence and absence, respectively, of 30% (w/v) PEG, and were normalized for plotting. For adjustment of pH values see Experimental Procedure. The data for the enzyme in solution were obtained by Dr. H. Gehring.

range examined (pH 5 to 9) the crystals of mitochondrial AspAT (pI $\sim$ 9.5 as determined by isoelectric focusing on thin-layer agarose gel, unpublished) behave as polycationic matrices expelling hydrogen ions and thus raising the pH value in their interior. This effect is expected to shift the pH/rate profile toward more acidic pH values.

Immobilization of several other enzymes in polycationic or polyanionic polymer matrices, has been observed to shift the pH/activity profile by more than one pH unit toward acidic or alkaline values, respectively (for a review, see Goldstein, 1976).

On the other hand, 30% (w/v) PEG in sodium phosphate buffered solutions displaces the pH values by about 0.25 pH unit toward the alkaline (Eichele et al., 1978; Atha and Ingham, 1981). Because the enzyme molecules in the crystals are in contact with buffer only, we have to assume that the pH value is 0.25 pH unit lower within the crystals than in the bulk solution resulting in an apparent shift of the pH/rate profile toward the alkaline, *i.e.* opposite to that expected from the polycationic matrix effect. Thus, the observed shift of the profile of microcrystalline AspAT toward the acidic by $\sim$ 0.5 pH unit (Fig. 8) must be the resultant of the two opposite effects, the gross matrix effect being minus 0.75 pH unit. A more specific local effect, *e.g.* alteration of the pK_a of a particular group due to a changed conformation of the enzyme in the crystal lattice, seems unlikely in view of the virtually unchanged shape of the shifted pH/activity profile with the difference between the crystalline enzyme and the enzyme in solution being constant over 3 pH units.

The previously observed shift of the pK_a' of enzyme-bound pyridoxal-5'-phosphate from 6.1 in solution to 6.4 in the crystal (Eichele et al., 1978) is opposite to the shift of the pH/activity profile. A possible explanation could be a specific local effect of the crystal lattice on the ionization behavior of the cofactor that is superimposed over the more general effects responsible for the shift of the pH/activity profile. Regardless of the mechanisms resulting in the shift of the pH/activity profile in the crystalline enzyme, it is clear that the shift is not the cause for its decreased catalytic activity.

3.6. Alanine Activity in Suspensions of Microcrystals and in Single Crystals

The assay conditions were the same as for the enzyme in solution except that the assay suspension contained 30% (w/v) PEG. The assays were stirred. Alternatively, the activity of single crystals was measured with a Zeiss UMSP 1 recording microspectrophotometer as described previously (Eichele et al., 1978). At zero time the crystals were immersed in 20% (w/v) PEG solution containing 400 mM alanine. As soon as the crystal had been placed in the light beam (after 1 to 2 min) the decrease in the absorbance at 355 nm was recorded. The measurements with single crystals were carried out at room temperature. The activity of the crystalline enzyme toward alanine is about the same as that of the enzyme in solution (Table I). In solution the reaction with alanine is about 10^4 times slower than that with aspartate. Suspensions of both thin and thick microcrystals as well as large single crystals show about the same activity, *i.e.* there is no diffusional rate limitation with this slowly reacting substrate.

The failure of the experiments with alanine and for this matter also with vinylglycine to detect the altered enzymic properties of crystalline AspAT emphasizes the importance of the use of fast-reacting natural substrates for assessing the functional consequences of the incorporation of the enzyme molecules into a crystal lattice.

3.7. Functional Nonequivalence of the Two Active Sites of the Aspartate Aminotransferase in the Crystalline State

Determination of the catalytic activity of the microcrystals as a function of substrate concentration reveals a biphasic behavior (Fig. 9) indicating the existence of two populations of active sites differing in their K_m' values by about one order of magnitude. The K_m' values

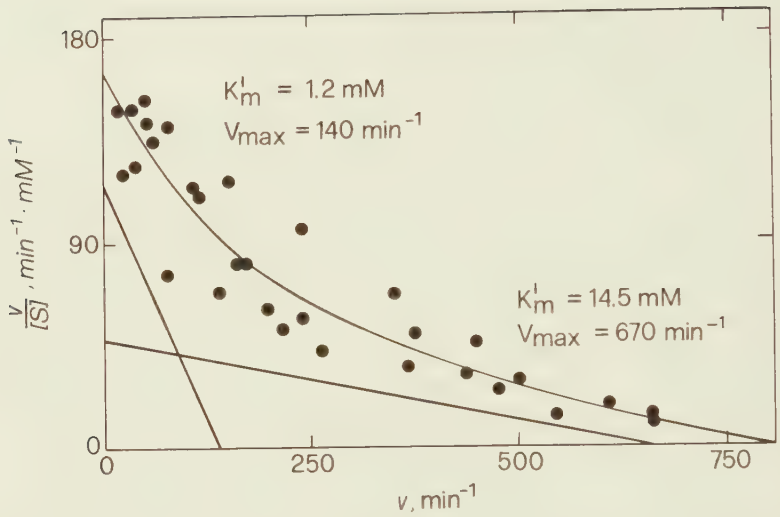


Fig. 9. Eadie-Hofstee plot for estimation of the catalytic parameters of crystalline mitochondrial AspAT. The apparent K_m value for 2-oxoglutarate (concentration varied from 0.2 to 100 mM) was determined at a constant 150 mM concentration of aspartate in PEG solution. In this plot three experiments are combined. Values for K_m' and V_{max} (calculated on the basis of the concentration of subunits) were estimated by nonlinear least squares fitting (Feldman, 1972). A similar biphasic plot was obtained when the concentration of aspartate was varied.

of the high affinity site for 2-oxoglutarate and aspartate are virtually the same as those of the enzyme in solution (Table II). The contribution of the high and low affinity site to the activity of the crystals is 1.5 and 7.5%, respectively, of that of the enzyme in solution. Assuming two different active sites in equimolar concentration (see below), their molar activities are 3 and 15% of that in solution. The measured overall activity per subunit of the crystalline enzyme (10%) closely corresponds with the mean of the contributions of the two subunits. A biphasic kinetic behavior with similar K_m' values was also found with microcrystals that had been crosslinked with glutaraldehyde. The values of V_{max} depend on the conditions used for crosslinking. The ratio of the values of the high and low affinity site of crosslinked crystals

TABLE II. Catalytic Parameters of Crystalline AspAT. The data were analyzed with Eadie-Hofstee plots (see Fig. 9).

	2-Oxoglutarate		Aspartate	
	K_m' (mM)	V_{max} (min^{-1})	K_m' (mM)	V_{max} (min^{-1})
Crystalline enzyme	1.2/14.5 [a]	140/670 [a]	0.5/5.5 [b]	140/430 [b]
Enzyme in solution	1.1 [c]	9000 [c]	0.3 [d]	9000 [d]

The values of V_{max} correspond to the molar activity per subunit.

[a] From Fig. 9

[b] Assayed in 0.2 to 150 mM aspartate at constant 20 mM 2-oxoglutarate, 30% (w/v) PEG, 50 mM sodium phosphate, pH 7.5.

[c] Assayed in 0.2 to 100 mM 2-oxoglutarate at constant 150 mM aspartate, 50 mM sodium phosphate, pH 7.5.

[d] Assayed in 0.1 to 100 mM aspartate at constant 50 mM 2-oxoglutarate, 50 mM sodium phosphate, pH 7.5.

is the same as for the non-crosslinked crystals. This result validates the assay procedure used for the non-crosslinked crystals that involves a centrifugation step in order to correct for the activity of dissolved enzyme.

The functional nonequivalence of the subunits of the crystalline enzyme is in contrast to the behavior of the enzyme in solution where both subunits of the dimer are kinetically equivalent. The enzyme in solution shows a strictly monophasic dependence of its activity as a function of the concentrations of both 2-oxoglutarate and aspartate (K_m' and V_{max} values are given in Table II). Detailed studies with the

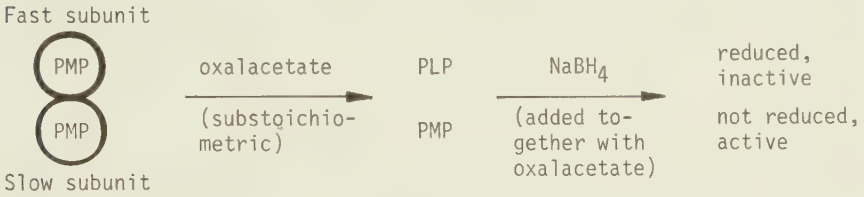
enzyme in solution such as the determination of the activity of hybrid dimers with one active and one inactive subunit (Schlegel and Christen, 1974, 1978; Lee and Churchich, 1975; Boettcher and Martinez-Carrion, 1975, 1976) and the examination of the active site occupancy pattern at transamination equilibrium (Schlegel et al., 1977) gave no indication for a functional nonequivalence of the subunits or functionally significant subunit interactions.

Like AspAT in solution, the pyridoxal form of the crystalline enzyme is readily inactivated by reducing the Schiff base between Lys 258 and pyridoxal-5'-phosphate with sodium borohydride (Hughes et al., 1962). Addition of cysteine sulfinate (final concentration 5 mM) to the crystal suspension completely prevented borohydride (two additions after 1 and 2 min of a 100 mM stock solution in 0.05 M NaOH, 30% (w/v) PEG, to give a final concentration of 5 mM) from abolishing the enzymic activity as measured both in an assay of the crystals and after dissolution of the crystals. Apparently, all active sites in the crystals had undergone the conversion to the pyridoxamine form. Addition of a keto acid (25 mM 2-oxoglutarate or 1 mM oxalacetate) to the washed suspension of microcrystals in the pyridoxamine form rendered them again susceptible to complete inactivation by sodium borohydride. After two additions of borohydride, an activity of about 2 to 5% in both the assay of the crystals and of the dissolved crystals was measured. Excess borohydride was trapped by adding pyridoxal-5'-phosphate and thus was prevented from consuming NADH in the assay solution. These experiments provide chemical support for the conclusion reached previously from spectroscopic evidence (Eichele et al., 1978) that in the enzyme crystals all active sites are catalytically competent.

The interpretation of the biphasic kinetic behavior as to indicate two nonequivalent active sites in the crystalline enzyme dimer is corroborated by selective mechanism-based elimination of either type of active sites. To a suspension of microcrystals in the pyridoxamine form oxal-

acetate was added in substoichiometric amounts such that only a fraction of all subunits were converted to the pyridoxal form. At the same time, excess sodium borohydride was added (Scheme III).

Scheme III. Preferential inactivation of fast subunits



The subunits undergoing transamination faster under these conditions will be inactivated preferentially by reduction of the internal aldimine. The preferential inactivation of the "fast" subunits manifests itself in a greater decrease in the activity of the suspension of the microcrystals than in the activity measured after dissolution of the crystals. In the crystals the "fast" subunits contribute to a higher degree to the measured activity whereas in solution all active sites are equivalent (Fig. 10). If the assumption of two different subunits is correct the converse experiment (Scheme IV) may be expected to result in the preferential inactivation of the "slow" subunits. Addition of limiting amounts of cysteine sulfinate to microcrystals in the pyridoxal form converts preferentially the "fast" subunits to the pyridoxamine form and thus preserves them from inactivation by subsequently added borohydride. The preferential escape of the "fast" subunits from inactivation is reflected in a smaller loss of activity of the crystals than of the dissolved crystals (Fig. 11).

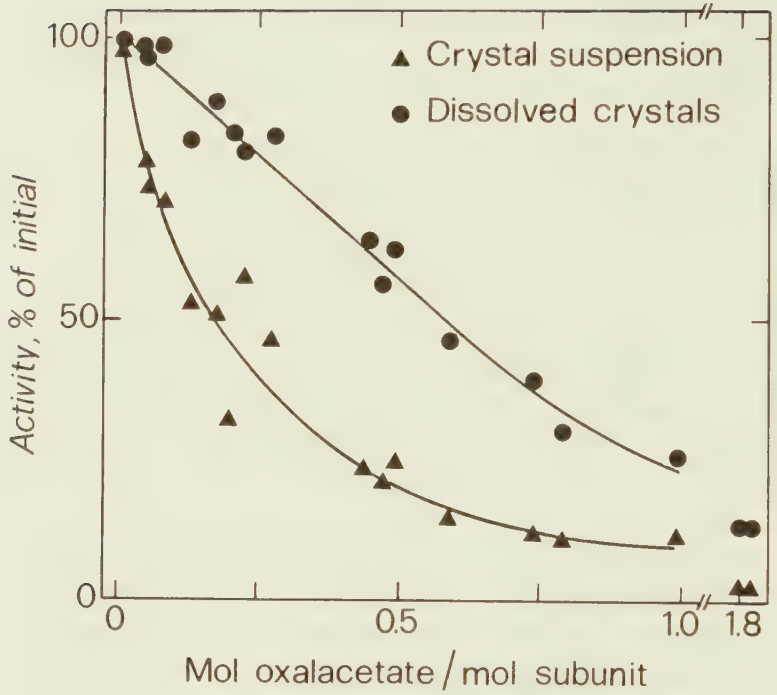


Fig. 10. Preferential inactivation of fast subunits. The plot combines three experiments. Microcrystals (pyridoxamine form, 0.1 to 0.4 mM subunit concentration) were kept in PEG solution. Sodium borohydride (final concentration 5 mM) and oxalacetate in the indicated concentrations were added simultaneously. The enzymic activity both of crystals and of dissolved crystals were assayed at 150 mM aspartate and 6 mM 2-oxoglutarate.

At the low concentrations of oxalacetate used the subunit with the lower K_m' would be expected to react faster, i.e. to be inactivated preferentially. This subunit has the lower molar activity (Table II) and contributes to a lesser degree to the total activity of the crystalline enzyme measured at high concentrations of substrates. Thus, in contrast to the experimental finding, the activity of the crystals would be expected to decrease less than the activity measured after dissolution of the crystals. The apparent discrepancy must be due to the different conditions used: low concentration of oxalacetate as the sole substrate in a one-

turnover reaction *vs.* high concentrations of an amino acid/keto acid substrate pair under steady state conditions.

The difference between the two subunits as revealed by this experiment appears to reflect a kinetic rather than a thermodynamic difference. A suspension of crystals in the pyridoxamine form was preincubated for 1 min with substoichiometric amounts of oxalacetate (0.5 mol oxalacetate/mol subunit) before adding borohydride. The ensuing decrease in activity of the crystals (36% of initial) is 1.5 times that measured after dissolution of the crystals (24% of initial). If borohydride is added together with oxalacetate the decrease in activity of the crystals (75%) is more pronounced, *i.e.* 2 times that measured with the dissolved crystals (37%).

Scheme IV. Preferential escape of fast subunits from inactivation

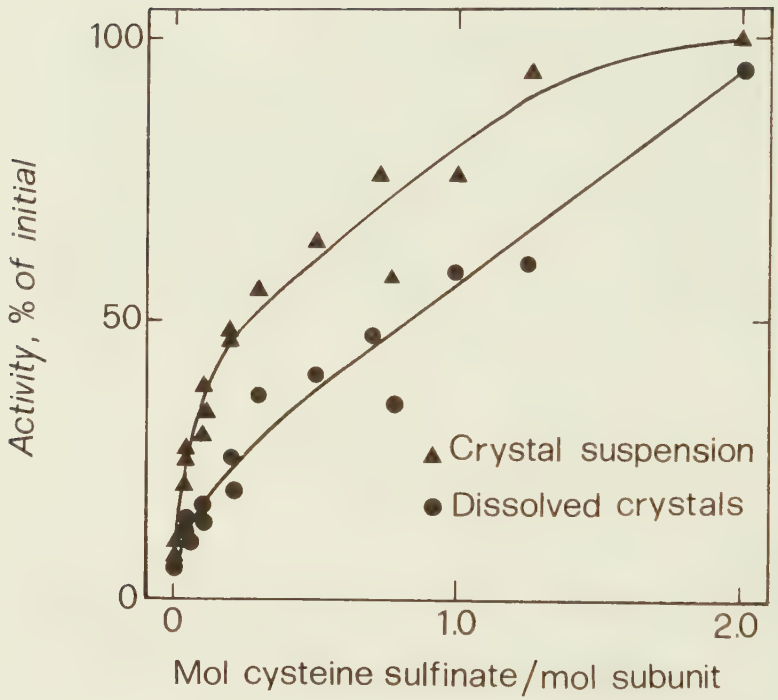


Fig. 11. Preferential escape of fast subunits from inactivation. The plot combines three experiments. Microcrystals (pyridoxal form, 0.1 to 0.4 mM subunit concentration) were kept in PEG solution. Cysteine sulfinate was added at the indicated concentrations. After 1 min, sodium borohydride was added (final concentration 5 mM). Enzymic activities were assayed as indicated in Fig. 10.

There are three possible explanations for the occurrence of two functionally nonequivalent active sites in the suspension of microcrystals: (1) Two different populations of microcrystals could exist in the suspension. This possibility is excluded by two lines of evidence. Scanning electron microscopy showed that all microcrystals were of the same habit. X-ray analysis of larger crystals grown under the same conditions showed

that they were isomorphous without exception (triclinic unit cell; J.N. Jansonius, personal communication). (2) In a crystal, two different populations of enzyme dimers could exist. A uniform distribution of two different dimers throughout the crystal is excluded because the unit cell of the crystals contains only one enzyme dimer (Eichele et al., 1979), *i.e.* in the crystal lattice each dimer has the same surroundings. The possibility that enzyme subunits at the surface of the crystals are less affected in their kinetic properties than those fully incorporated in the crystal lattice appears to be excluded by the following consideration. The average thickness of the crystals comprises about 80 layers of enzyme dimers (Eichele et al., 1979). Because the dimers are arranged with their longest dimension nearly perpendicular to the main faces of the crystals (Eichele et al., 1979; Eichele, 1980), the two main surfaces are formed by one type of subunit only (S or S*, respectively, Ford et al., 1980). Thus, the subunits of one of the two surface layers could account for a fraction of the measured activity corresponding at most to 1/160, *i.e.*, to 0.6%, of the activity of the enzyme in solution. The molar activities of the high affinity active sites whose K_m' is the same as that of the enzyme in solution (Table I) is 1.5%, however. (3) The best explanation of the present data assumes that in the crystal the two subunits of the enzyme dimer have become kinetically different from each other. This notion is supported by X-ray crystallographic data. The two subunits obey a molecular dyad; however, they differ in their lattice contacts (Eichele et al., 1979). Apparently, the different surroundings give rise to different kinetic properties of the two subunits that are functionally equivalent in solution and proved conformationally identical in X-ray analysis of the unliganded enzyme (Ford et al., 1980). A similar lattice-induced asymmetry has been reported for the reaction of ferrihemoglobin with azide that is monophasic in solution and becomes biphasic in the crystal. This finding has been explained by lattice-induced functional asymmetry of the subunits of the hemoglobin tetramer (Chance and Ravilly, 1966).

How does the asymmetric packing give rise to the observed functional asymmetry of the AspAT dimer? The dissimilar lattice forces either result in a small difference in conformation that is not detected by X-ray analysis at the present level of resolution or induce a difference in the conformational adaptability of the two subunits. A lattice-induced asymmetry in the conformational adaptability of the two subunits has indeed been found by X-ray crystallographic analysis of the phosphopyridoxyl aspartate apoenzyme complex. Introducing N-(5'-phosphopyridoxyl)-L-aspartate, an analog of the covalent coenzyme-substrate intermediates, into apoenzyme crystals led to a marked conformational change in only one of the two subunits, *i.e.* in the so-called S* subunit (Eichele et al., 1979; Eichele, 1980; Ford et al., 1980). N-(5'-Phosphopyridoxyl)-L-aspartate occupies the S and S* subunit to different degrees. The subunit S is occupied only to 40 to 50% under conditions where the subunit S* is fully liganded (Eichele, 1980). The liganded subunits are catalytically inactive. The unequal binding of the analog allows a tentative identification of the low and high affinity subunits with the S and S* subunits, respectively. Suspensions of microcrystals of AspAT in the apoenzyme form were incubated with the analog. In timed intervals samples of the crystal suspension were transferred into PEG solutions containing pyridoxal-5'-phosphate. Subsequently, the enzymic activity of the crystals and of the enzyme after dissolution of the crystals was measured (Fig. 12). The loss in activity of the crystalline enzyme was less than that of the dissolved crystals. Apparently, N-(5'-phosphopyridoxyl)-L-aspartate is bound preferentially by those subunits that contribute less to the enzymic activity of the crystals. The enzymic activities were measured under saturating substrate concentrations, such that on the basis of the catalytic parameters of the two active sites (Table II) the low affinity site is expected to contribute 3/4 of the total measured activity. Thus, the S and S* subunit correspond with the low and high affinity subunit, respectively.

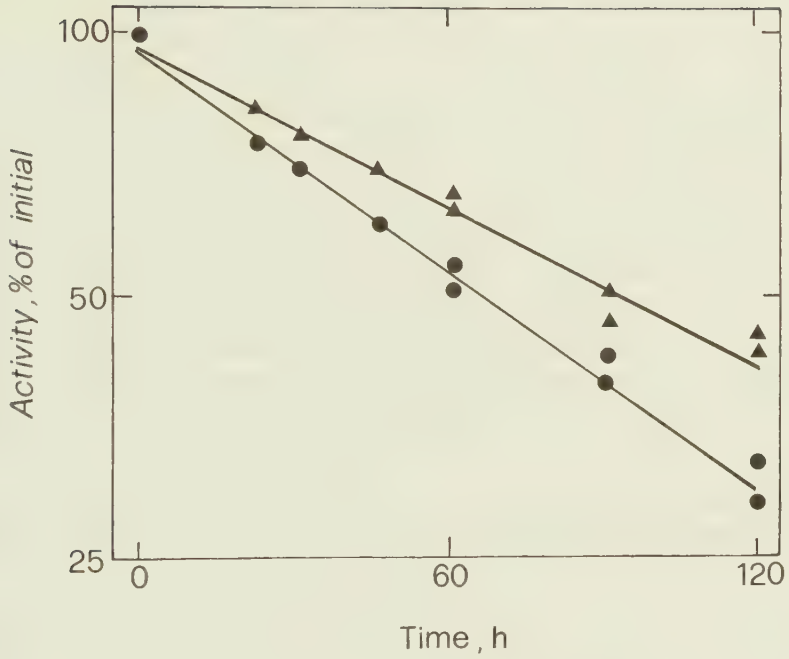


Fig. 12. Differential binding of N-(5'-phosphopyridoxyl)-L-aspartate to the high and low affinity subunit. Suspensions of microcrystals of AspAT in the apoenzyme form (0.14 mM) were incubated in a PEG solution containing 0.5 mM N-(5'-phosphopyridoxyl)-L-aspartate. At the indicated times, samples were reconstituted with 2 mM pyridoxal-5'-phosphate for 10 min. The activity of the crystals was measured in assay PEG solution (▲). The activity after dissolution of the crystals was measured in an assay without PEG (●). The activities were measured under saturating substrate concentrations; the assay PEG solution contained 50 mM aspartate and 100 mM 2-oxoglutarate, while the assay solution without PEG contained 50 mM aspartate and 50 mM 2-oxoglutarate. The measured activities were independent of the duration of the incubation with pyridoxal-5'-phosphate (control up to 18 h). All experiments were carried out at 25°C.

4. Concluding Remarks

The studies with microcrystals of AspAT emphasize the importance of a detailed kinetic analysis for the assessment of the effects of the crystal lattice on the functional properties of the enzyme. A prerequisite for obtaining significant data are the exclusion of diffusional rate limitation and the use of fast-reacting natural substrates. For example, the experiments with the very slowly reacting substrate alanine and the k_{cat} inhibitor vinylglycine failed to reveal the decreased catalytic efficiency and the functional asymmetry of the crystalline enzyme. The experimental conditions under which the enzymic activity of crystalline AspAT was measured warrant that any observed difference in the catalytic parameters to those of the enzyme in solution can be unequivocally attributed to crystal packing effects. A direct effect of the crystallization medium on the enzyme activity is precluded because PEG of higher molecular weight does not penetrate the interior of the crystals (McPherson, 1976); the use of non-crosslinked crystals obviates interference of modification of side chains. The decrease in activity by about one order of magnitude in the crystalline enzyme must be due to lattice forces either distorting the geometry of the active site or impeding ligand-induced and syncatalytic conformational adaptations which are integral features of the catalytic mechanism. The different lattice contacts of the conformationally identical subunits of dimeric AspAT in triclinic crystals give rise to different functional properties of the two subunits. In solution, the two subunits are functionally equivalent and in the crystals of the unliganded enzyme no structural asymmetry of the dimer is detectable by high resolution X-ray crystallographic analysis (Ford et al., 1980). Thus, the functional asymmetry of the crystalline enzyme dimer very likely is due to a difference in the contraints exerted by lattice forces on the conformational flexibility of the two subunits. The coincidence of low and high affinity for the substrate with high and low molar activity, respectively, suggests that in the low affinity subunit part of the binding energy of the substrate is used for a conformational change resulting in a higher reaction rate of this subunit.

III LINEAR DICHROISM STUDIES WITH SINGLE CRYSTALS OF ASPARTATE AMINOTRANSFERASE

1. Introduction

X-ray crystallographic analysis of AspAT has shown that the orientation of the coenzyme in the active site changes from one functional state to the other (Eichele, 1980; Ford et al., 1980). For example, the coenzyme ring in the pyridoxamine form is tilted forward by about 15° in comparison to its orientation in the pyridoxal form; in the complex of apoenzyme with N-(5'-phosphopyridoxyl)-L-aspartate, an analog of the covalent coenzyme substrate intermediates, the tilt angle is even about 30°.

The linear dichroism studies of single crystals of AspAT are based on the fixed spatial orientation of the transition dipole moments related to near-UV absorption bands of the coenzyme chromophores. The absorption of plane-polarized light thus depends on the orientation of the crystal with respect to the plane of polarization. The absorption is due to π-π^* transitions of which the transition dipole moments lie in the ring plane of the coenzyme. If the direction of the transition dipole moment in the coenzyme molecule is known, changes in the spatial orientation of the coenzyme ring may be detected by linear dichroism measurements. Such measurements allow to assess the spatial orientation of the chromophore in each intermediate that is characterized by a specific absorption band. If the orientation of the absorbing species in the crystal is known from X-ray crystallographic analysis, linear dichroism measurements may serve to determine the direction of the transition dipole moment within the chromophoric molecule and thus to verify quantum-chemical predictions. Similar applications of linear dichroism measurements are also possible with noncrystalline oriented samples such as enzymes immobilized in

stretched plastic foils or compressed polyacrylamide gels (for a review, see Hofrichter and Eaton, 1976).

In the linear dichroism experiments plate-like crystals were rotated in a plane perpendicular to the light beam. The orientation of the crystal with respect to the plane of polarization corresponding to maximum absorption and the polarization ratio were determined. The latter is defined as the ratio of maximum absorption (A_{max}) to minimum absorption (A_{min}) as measured at an orientation of the crystal perpendicular to that corresponding to A_{max}. The present study discusses the experimental findings in a qualitative manner. The interpretation given is based on both quantum-chemical calculations of the direction of the transition dipole moment of the coenzyme in different transamination intermediates (Savin et al., 1973; Song, P.-S., personal communication to Metzler, D., and Christen, P.) and the spatial orientation of the coenzyme in the active site as deduced from X-ray crystallographic analysis (Eichele, 1980). The crystals used for both the X-ray crystallographic analysis and the linear dichroism measurements are of space group P1 and contained one enzyme dimer per unit cell. Thus, there are two differently oriented transition dipole moments of the coenzyme chromophore in these crystals. Previous microspectrophotometric analyses of single crystals of AspAT have shown that in a crystal all active sites undergo reversible transamination on addition of the appropriate substrates (Eichele et al., 1978). The different intermediates of enzymic transamination are characterized by specific absorption bands (Braunstein, 1973; Metzler, 1979). This circumstance allows to assess their linear dichroism parameters even if at a given condition, *e.g.* at transamination equilibrium, several intermediates co-exist. Linear dichroism measurements may thus complement X-ray crystallographic analysis which does not allow to differentiate between co-existing intermediates due to the averaging of the electron density values over time and space.

2. Experimental Procedure

Materials. Mitochondrial AspAT was isolated from chicken heart (Gehring et al., 1977a). The specific activity of the enzyme preparations was 200 U/mg (*Section II*; Kirsten and Christen, 1982). DL-*erythro*-3-Hydroxyaspartate was synthesized according to Kornguth and Sallach (1960). Polyethylene glycol 6000 synthetic grade was purchased from Merck.

Equipment. A Zeiss UMSP I recording double beam microspectrophotometer was used. A polarizing filter (105 UV from Polacoat, Cincinnati Ohio, USA) was placed right after the luminous field stop. The absorption spectra of the crystals were corrected by the absorption of the medium surrounding the crystals. The diameter of the measured light beam was 32 μm. The measuring diaphragm was placed right in front of the photomultiplier (Fig. 13). Polarization ratios were determined with a Zeiss single beam recording microscope photometer (MPM 03 UV), equipped for plane-polarized light. The diameter of the measured light beam again was 32 μm. The vertical light beam of the two instruments allowed convenient horizontal mounting of the crystals. In both instruments an objective Ultrafluar 32, aperture 0.4, was used. The orientations of the crystals were documented photographically with the built-in cameras.

Crystallization. Microspectrophotometric measurements of the coenzyme absorption especially in the near-UV region require a correction for the absorption of the protein moiety. Therefore, the measurements were performed on the reconstituted half of an apoenzyme crystal with the other half serving as a blank.

Single crystals of apoAspAT were obtained using the *"hanging drop"* version of the crystallization procedure described by McPherson (1976). The crystallization of mitochondrial AspAT from chicken has been reported earlier (Gehring et al., 1977a). A fresh preparation of apoAspAT (Schlegel and Christen, 1974) in 10 mM sodium phosphate, pH 7.5, was

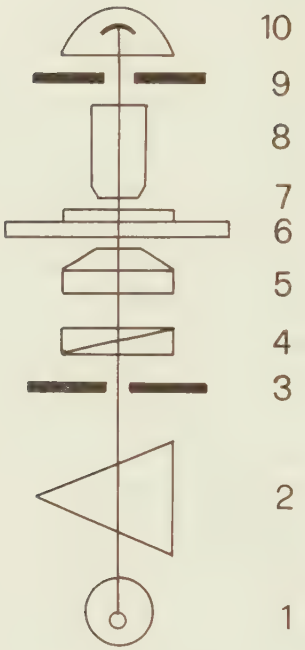


Fig. 13. Course of the light beam in the UMSP I and the MPM 03 microspectrophotometer: 1 light source, 2 monochromator, 3 luminous field stop, 4 polarizer, 5 condenser, 6 revolving stage, 7 specimen, 8 objective, 9 measuring diaphragm, 10 photomultiplier.

concentrated by ultrafiltration (Amicon PM10 membrane) to 10 mg/ml. The enzyme solution (10 µl) was mixed with an equal volume of a PEG containing solution (final concentrations 8 to 24% (w/v) PEG, 10 mM sodium phosphate, pH 7.5) on a silanized microscope coverslip. For silanization the coverslips were held for 20 min in 5% (v/v) dimethyldichlorsilane in carbon tetrachloride at room temperature. Subsequently, they were extensively washed with water and kept at 60°C in a drying closet for 2 h. The coverslip with the drop in hanging position

was placed on top of a well of an assay plate (96-well monoclonal assay plate from Flow Laboratories). Each well contained 1 ml of the same PEG solution as used for mixing and was sealed with immersion oil. The assay plates were kept at 17°C in the dark. Crystals appeared within a few days. The crystals of apoAspAT are isomorphous with those of the enzyme liganded with pyridoxal-5'-phosphate (Eichele et al., 1979). Plate-like crystals with dimensions 300 x 200 x 10 to 40 µm were selected and transferred into glass vials (17 x 15 mm) containing 0.5 ml 20% (w/v) PEG, 10 mM sodium phosphate, pH 7.5. The medium was changed once. The crystals were transferred with a thin-walled glass capillary for X-ray crystallographic purpose (according to Debye-Scherrer from W. Müller, Berlin) under a stereomicroscope (magnification 10 to 80 x).

Reconstitution of apoenzyme crystals; formation of enzyme-substrate intermediates. Plate-like crystals of apoAspAT were cut in half with a fine tungsten needle [a]. One half was transferred into 20% (w/v) PEG, 10 mM sodium phosphate, pH 7.5, 1 mM pyridoxal-5'-phosphate and kept for 1 h at room temperature in the dark. The reconstituted crystals were washed with 4 changes of the same PEG solution over a period of 1 to 2 days. The washed reconstituted crystals and the corresponding apocrystals were kept separately at 4°C in the dark. Measurements were performed within a few days. The degree of reconstitution was examined by measuring the specific activity of the enzyme after dissolution of a few crystals. In all experiments the specific activity correspond with the original value. Full reconstitution of apoenzyme crystals was also demonstrated by X-ray crystallography (Eichele et al., 1979).

The different enzyme substrate intermediates and adsorption complexes of the crystalline enzyme were formed by soaking both the apo and holo half of a crystal in 500 µl of 20% (w/v) PEG, 50 mM sodium phosphate,

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[a] A piece of tungsten wire (3 cm) was inserted (about 0.5 cm) in the molten end of a glass tube. Sodium nitrite was melted in a metal crucible. The tungsten wire was dipped into the molten mass and pulled through repeatedly until a sharp tip had been formed (G. Eichele, personal communication).

pH 7.5, (*PEG solution*) containing the appropriate substrate analog. The crystals did not suffer any damage on transfer into the substrate containing PEG solution. Occasionally, soaking in *erythro*-3-hydroxy-aspartate generated little cracks in the holocrystal that, however, did not interfere with the spectroscopic measurements.

Measuring procedure. After soaking the crystals in the substrate containing PEG solution for 5 min at room temperature, they were placed on a quartz slide in a fresh drop of the same solution. The mounting of the crystals for microspectrophotometry was as described elsewhere (Eichele et al., 1978). The slide was put on the horizontal revolving stage of the microspectrophotometer. If not otherwise indicated, the crystals were placed onto the quartz glass slides on the main faces (001) or (00$\bar{1}$). In all cases, with the exception of the experiment with *erythro*-3-hyroxyaspartate, the reaction equilibrium had been reached before the time of recording the spectra as indicated by time-independent absorption values. The centric position of the crystal with respect to the vertical light beam of the microspectrophotometer and the homogeneity of the measured area was checked through the built-in microscope. In order to obviate interference by an anisotropic response of the photomultiplier, the polarizer was kept in a fixed orientation. The rotation of the plane of polarization by the optically active protein might influence the determination of the linear dichroism parameters. In assessing the extent of optical activity which is higher in the near-UV than in the visible region, the experimental parameters of the pyridoxamine form at 333 nm were determined with thick crystals (40 to 80 μm; A_{333} from 0.69 to 1.56). The measured orientation angle of the crystal giving maximum absorption at fixed wavelength and the polarization ratio were found to be independent of the thickness of the crystals. The validity of the Lambert-Beer law was established for crystals at a fixed orientation (Fig. 14).

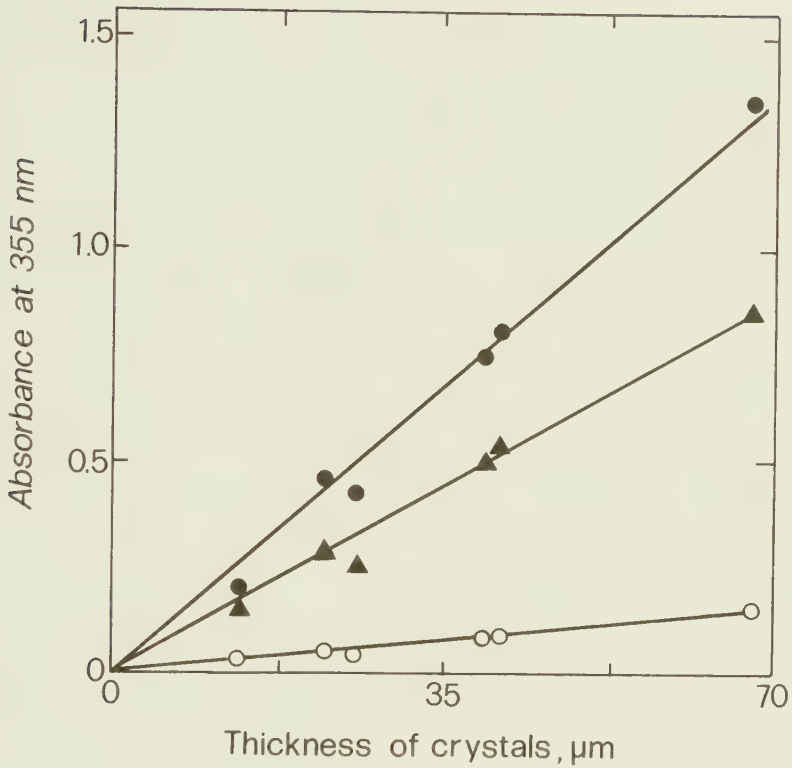


Fig. 14. Validity of the Lambert-Beer law for identically oriented crystals. The crystals were immersed in PEG solution (pH 7.5) and the absorption was measured at 355 nm. Crystals were oriented such that the plane of polarization was either parallel (●) or perpendicular (○) to their a-axis (see Fig. 16).

The same crystals were then transferred into 20% (w/v) PEG, 50 mM sodium acetate, pH 5.1, and oriented with their a-axis parallel to the plane of polarization. The absorption was measured at 355 nm (▲); if the a-axis is perpendicular to the plane of polarization, the absorbance values coincide with those measured at pH 7.5. The thickness of the crystals was determined after crosslinking with 1.5% (w/v) glutaraldehyde in PEG solution for 1 h at room temperature. For measuring, the crystals were tilted onto their narrow side and kept in this position by a little bit of vacuum grease. A stereomicroscope equipped with a calibrated micrometer scale was used.

3. Results and Discussion

The dichroic behavior of the coenzyme chromophore was examined by rotating a crystal in a plane perpendicular to the plane of polarization and measuring the absorbance as a function of the orientation of the crystal. The wavelengths tested correspond to the absorption maxima of the different transamination intermediates (see Fig. 16). The orientation for A_{max} and A_{min} at a given wavelength are related by a rotatory angle of 90° with a periodicity of 180° (Fig. 15).

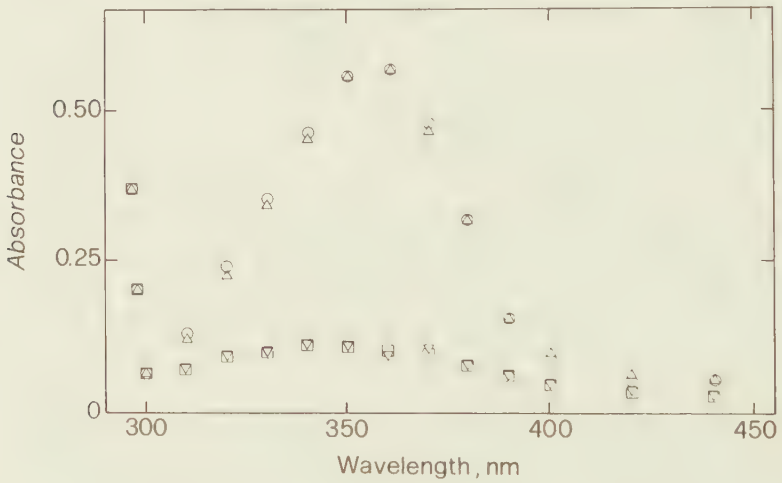
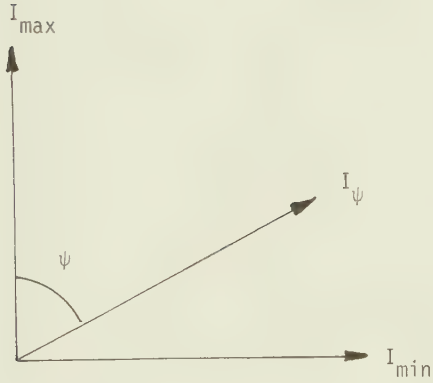


Fig. 15. Polarized absorption spectra of an enzyme crystal in the pyridoxal form. The crystal was rotated by 360° in a plane perpendicular to the plane of polarization and the spectra were recorded in the orientations for A_{max} (○,△) and for A_{min} (□,▽) at 355 nm. The orientations for A_{max} and for A_{min} are related by 90° with a periodicity of 180°. The spectra are corrected for the contribution of the apoenzyme half of the crystal (see under "[illegible]"), which in this wavelength region did not show any change in absorption on rotation.

In optically anisotropic crystals the incident light is transmitted as two independently vibrating waves polarized in orthogonal planes (Slayter, 1970). In triclinic crystals, like those of AspAT, the orientation of these planes is wavelength-dependent and does in general not coincide with the crystallographic axes. On rotation of such a crystal around the beam direction, the transmittance at a fixed wavelength varies as a function of the square of the cosine of the revolution angle:



I_{max} Orientation of crystal for maximum transmittance

I_{min} Orientation of crystal for minimum transmittance

I_{ψ} Orientation of plane of polarization of incident light beam

$$\text{Transmittance } I_{\psi} = I_{max}\cdot\cos^2\psi + I_{min}\cdot\sin^2\psi$$

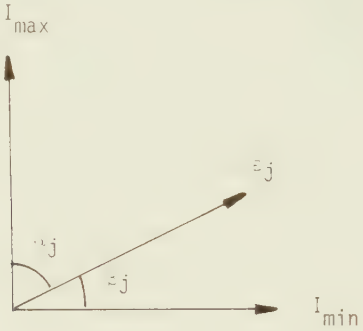
$$= I_{max}\cdot\cos^2\psi + I_{min}\cdot(1-\cos^2\psi)$$

$$= I_{min} + (I_{max} - I_{min})\cdot\cos^2\psi$$

The measured transmittance relates to the projection of the transition dipole moments onto the two orthogonal vibration directions of the plane waves.

In the present treatment we assume that the orientation of maximum and minimum absorption in the main face (001) of our plate-like crystals correspond to the vibration directions of the two plane waves. Accord-

ingly, the projections of the transition dipole moments (ε_j) onto these directions determine the polarization ratio:



$$I_{max} = I_o \cdot 10^{-A_{min}} = I_o \cdot 10^{-(\Sigma_j \varepsilon_j^2 \cos^2 \alpha_j)dc}$$

$$I_{min} = I_o \cdot 10^{-A_{max}} = I_o \cdot 10^{-(\Sigma_j \varepsilon_j^2 \cos^2 \beta_j)dc}$$

(d, optical path;
c, concentration)

For more information on the determination of the extinction directions and the dependence of the absorption values on the orientation angle, the reader is referred to the publications of Martin et al., (1976, 1979).

Single crystal absorption spectra of different functional states of the enzyme obtained in non-polarized light are in most cases virtually superimposable on those of the enzyme in solution. The observed differences have been explained by lattice effects interfering with the position of the transamination equilibrium (Eichele et al., 1978). In plane-polarized light the crystals showed absorption maxima at the same wavelength.

In order to monitor the dynamic behavior of the coenzyme during the successive stages of the half-cycle of transamination, the following functional states of the enzyme (see Scheme II) were investigated: the pyridoxal enzyme at pH 7.5 (λ_{max} 355 nm) and its protonated form at pH 5.1 (λ_{max} 430 nm, 355 nm), the nonproductive adsorption complex of the pyridoxal enzyme with a keto acid substrate such as 2-oxoglutarate or oxalacetate (λ_{max} 430 nm, 355 nm), the external aldimine formed in the presence of the substrate analog 2-methylaspartate (λ_{max} 430 nm, 355 nm), the semiquinonoid intermediate that accumulates in the presence of *erythro*-3-hydroxyaspartate (λ_{max} 495 nm, 333 nm, 355 nm), the pyridoxamine enzyme formed on addition of the amino acid substrate cysteine sulfinate (λ_{max} 333 nm), and the enzyme at transamination equilibrium in the presence of aspartate and oxalacetate (λ_{max} 365 nm, 333 nm). Furthermore, complexes of the apoenzyme with N-(5'-phosphopyridoxyl)-L-aspartate (λ_{max} 333 nm), N-(5'-phosphopyridoxyl)-L-alanine (λ_{max} 340 nm), and N-(5'-phosphopyridoxyl)-3-iodo-L-tyrosine (λ_{max} 333 nm) were investigated as analogs of covalent enzyme-substrate intermediates. The complexes of the apoenzyme with phosphopyridoxyl amino acids have also been analyzed by X-ray crystallography (Eichele et al., 1979; Eichele, 1980).

In the following, the full experimental data on three examples of functional states of the enzyme, *i.e.* (1) the unprotonated pyridoxal form, (2) the semiquinonoid intermediate, and (3) the pyridoxamine form, will be presented together with their qualitative interpretation. The data of the other functional states are summarized in Table III in the APPENDIX. The three functional states to be discussed are characterized by typical absorption bands (Fig. 16). As is evident from the micrographs of Fig. 16, the orientation of the crystal corresponding to A_{max} at these absorption bands differs from one functional state to the other. The absorption band of the pyridoxal enzyme at 355 nm almost completely disappears when the crystal is rotated by 90° away from the orientation corresponding to A_{max}. The measured polarization ratio $A_{max}/A_{min} = 25$

Fig. 16. Polarized absorption spectra of a single crystal of AspAT in three different functional states. The plate-like crystal was placed on the main face (001). The propagation of plane-polarized light was perpendicular to the parallel (001) and (00$\bar{1}$) faces of the crystal. After recording the spectra of the pyridoxal form, the crystal was soaked in 500 µl PEG solution containing 20 mM DL-*erythro*-3-hydroxy-aspartate and the spectra of the ensuing semiquinonoid intermediate were recorded. The same crystal was then transferred into 500 µl of a PEG solution containing 5 mM cysteine sulfinate and thus converted to the pyridoxamine form. Spectra were taken 5 to 10 min after transfer of the crystal into the respective substrate solutions. The micrographs document the orientation of the crystal giving A_{max} at the indicated wavelengths in the given functional states. The propagation direction of the polarized light is perpendicular to the plane of the micrograph. The orientation angle α is read between the marked a-axis and the vertical onto the plane of polarization. The vertical coincides with the reticule of the microscope and the horizontal edge of the micrograph. The numerical data are summarized in Table IV.

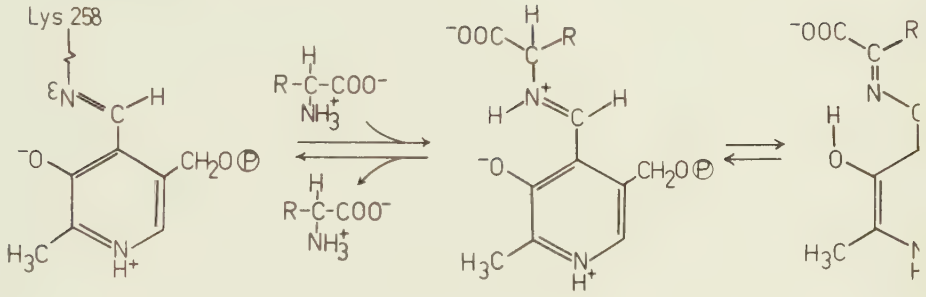
(Table IV) is remarkably high. Such a high value would be expected if the two transition dipole moments were in a plane perpendicular to the main face (001) of the crystal which intersects (001) in a direction close to that of maximum absorption. In our triclinic crystals, the transition dipole moments of the two coenzymes of the enzyme dimer are related by a molecular dyad. Therefore, they are oriented differently in space. However, in the pyridoxal form, the transition dipole moment of one of them is probably oriented such that the contribution of one coenzyme markedly exceeds that of the other. This interpretation is based on X-ray crystallographic data and quantum-chemical calculations of the orientation of the transition dipole moment in the plane of the pyridine ring of the coenzyme (see below).

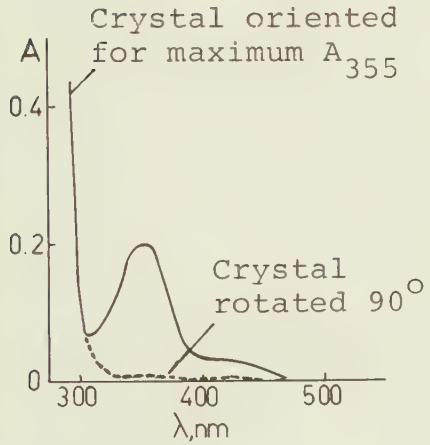
In the presence of *erythro*-3-hydroxyaspartate when the semiquinonoid intermediate (λ_{max} 495 nm) is accumulated, the orientation of the crystal corresponding to A_{max} differs quite markedly from that of the crystal in the pyridoxal form. The 495 nm absorption band exhibits a similarly high polarization ratio as that of the pyridoxal form at 355 nm

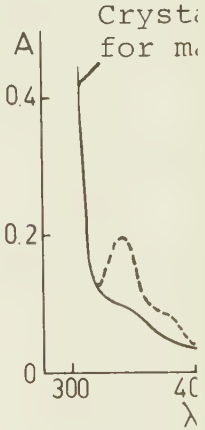
Pyridoxal form
(internal aldimine)

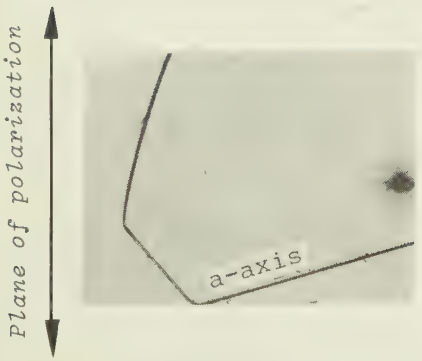
External
aldimine

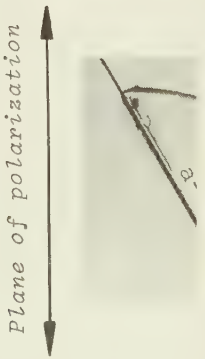
Semiqu
interm







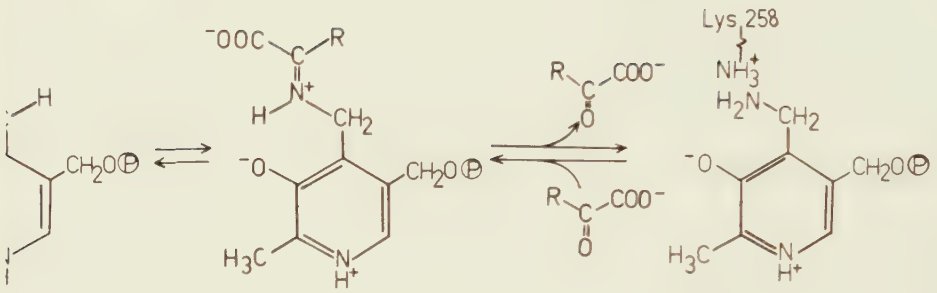




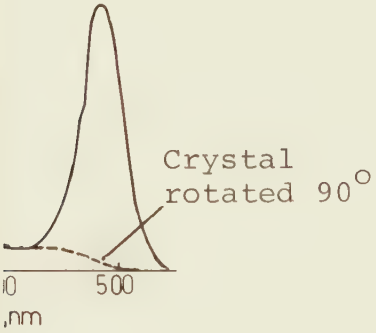
inoid
ediate

Ketimine

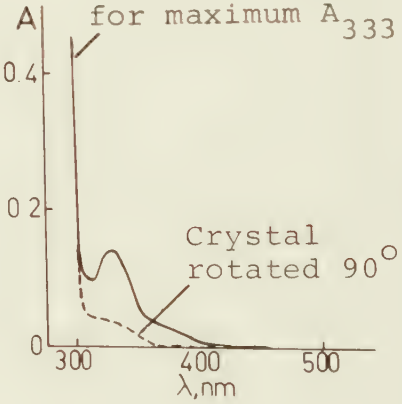
Pyridoxamine form



al oriented
aximum A_{495}



Crystal oriented
for maximum A_{333}



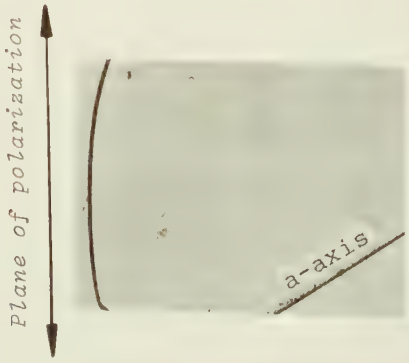


TABLE IV. Linear dichroism data of transamination intermediates of AspAT in triclinic crystals (space group P1), a summary of experiments performed as described in Fig. 16.

Transamination intermediates	λ_{max} (nm)	orientation angle $\alpha \pm$ S.D.[a]	Polarization ratio A_{max}/A_{min} [b]
Pyridoxal form			
at pH 7.5	355	14.3 ± 2.7° (N=29)	25.4 ± 5.4 (N=11)
at pH 5.1 [c]	430	17.0 ± 3.8° (N=11)	10.3 ± 1.9 (N= 6)
	355	N.D.	
Semiquinonoid intermediate	495	- 58.5 ± 4.5° (N=13)	27.7 ± 3.6 (N= 3)
	333	∿ 32°	
	355(sh)	2 to 22°	
Pyridoxamine form	333	36.0 ± 4.4° (N=17)	4.4 ± 0.7 (N= 4)

[a] The values obtained with the UMSP I and the MPM 03 photometer are combined. The sign given applies to the case when the crystal is placed on its (00$\bar{1}$) face.

[b] The polarization ratios were determined with the MPM 03 photometer (see *"Equipment"*). The contribution of the apoenzyme crystal was subtracted both from A_{max} and A_{min}.

[c] In 20% (w/v) PEG, 50 mM sodium acetate, pH 5.1.

(Table IV). Part of the enzyme exists in the pyridoxamine (λ_{max} 333 nm) and the pyridoxal form (λ_{max} 355 nm). As it happens, the absorption maxima of these bands are reached when the crystal is rotated by ~ 90°, *i.e.* the orientation of the crystal corresponding to A_{max} at 495 nm is about the same as that for A_{min} at 333 and 355 nm. The absorption at 495 nm decreases with time ($t_{1/2}$ = 330 min) because *erythro*-3-hydroxyaspartate reacts slowly to produce the pyridoxamine form of the enzyme (λ_{max} 333 nm). As expected the orientation angles α read for A_{max} at 355 nm and at 333 nm in the presence of *erythro*-3-hydroxyaspartate are not significantly different from those determined for the pyridoxal form (at 355 nm) and the pyridoxamine form (at 333 nm) in the absence of the substrate analog (Table IV).

The interpretation of the data is based on the following information: (1) The directions of the transition dipole moments of the various absorption bands. These were estimated for the different transamination intermediates by quantum-chemical calculations performed independently by P.-S. Song (personal communication to D. Metzler and P. Christen) and F.A. Savin et al. (1973, and personal communication to P. Christen). All transition dipole moments lie in the pyridine ring plane. Very similar results were obtained by the two methods used, *i.e.* the Pariser-Parr-Pople approximation by Song, and the CNDO/S approximation by Savin. The accuracy of both methods is estimated to be 10 to 30°. The calculations of Song showed only relatively small changes in the directions of the transition dipole moment for most of the functional states, except for the semiquinonoid intermediate. (2) The spatial orientations of the coenzyme rings as estimated by X-ray crystallographic analysis of the pyridoxal form of the enzyme at 2.8 Å resolution (Eichele, 1980). Of each type of crystals used in linear dichroism measurements one specimen was analyzed by X-ray diffraction and several were inspected by conoscopy (performed by G. Eichele) in order to establish that they were of the same habit as the larger crystals used for the high resolution structural analysis. The two coenzyme rings in the pyridoxal form of the crystal lie

in planes that are almost parallel to the plane containing the molecular dyad and being perpendicular to the (001) face, *i.e.* the plane of projection of the molecular dyad onto the (001) face (Fig. 17). This fact permits the qualitative interpretation of the linear dichroism data of crystalline AspAT in the pyridoxal form. The approximation that the coenzyme rings lie in the plane of projection of the molecular dyad is a justified simplification (Fig. 18).

The reorientations of the transition moments may also be described on the basis of an intrinsic reference system, *e.g.* the angle δ between the direction corresponding to maximum absorption and the trace of the molecular dyad on the (001) face (Fig. 19A). The relationship between angle δ and the orientation angle α (Fig. 16) is explained in Fig. 19B. While the angle δ is small in the pyridoxal form (λ_{max} 355 nm) and the pyridoxamine form (λ_{max} 333 nm) (Figs. 19A and B), it is -71° in the semiquinonoid intermediate (λ_{max} 495 nm). Changes in the orientation of the crystal required for obtaining maximum absorption not necessarily indicate a movement of the coenzyme ring out of its original plane in a particular intermediate because of the wavelength dependence of the vibration directions of the two plane waves. As already stated, in the pyridoxal form the two coenzyme rings are positioned almost parallel to the plane of projection of the molecular dyad. The direction of the transition dipole moments of the two coenzyme rings with respect to the electric vector of the polarized light is such that one of the two chromophores contributes significantly more to the measured absorption than the other one (Fig. 18). For the other two functional states the individual contributions of the two chromophores to the measured absorption cannot be estimated because the tilt angle of the coenzyme rings are unknown. The angle δ (see Figs. 19A and B) does not specify the tilt angle.

An estimation of the tilt angles requires additional information, *i.e.* the polarization ratio and a complementary set of linear dichroism data on crystals examined in a second direction. For these measurements

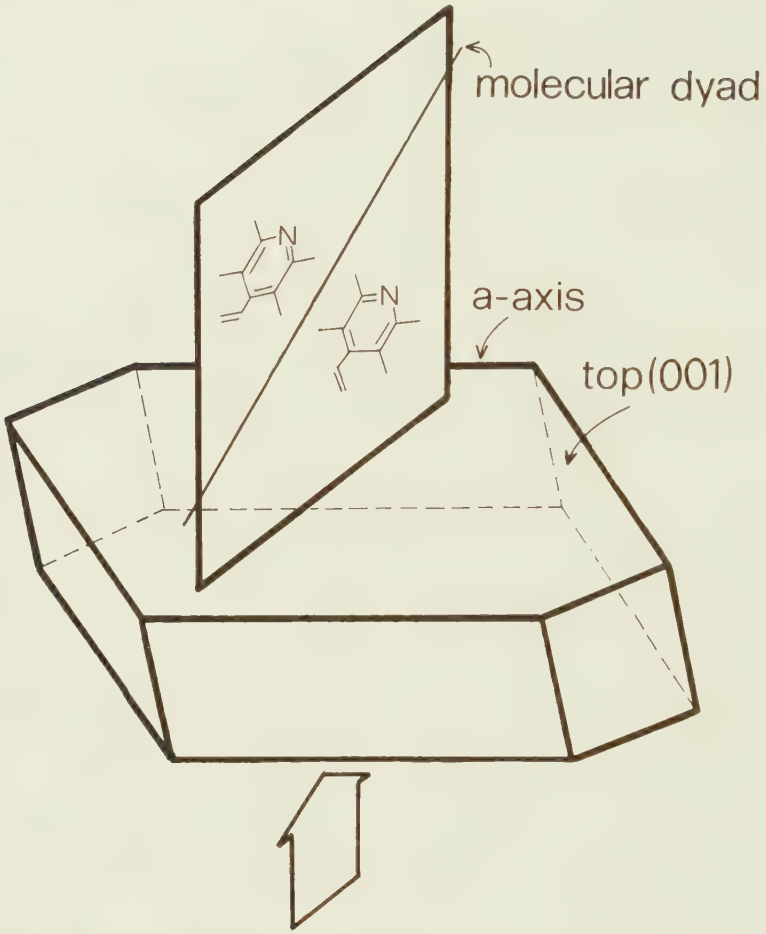


Fig. 17. Orientation of the coenzyme rings in the crystal. The top face of the crystal is designated by the Miller indices (001); the a-axis of the triclinic unit cell coincides with the usually longest edge of the crystal. In the pyridoxal form of the enzyme the two coenzyme rings lie in planes that are almost parallel to the plane of projection of the molecular dyad onto the (001) face. The angle of intersection of the coenzyme containing planes and the projection plane of the molecular dyad is about 2° (J.N. Jansonius, personal communication). The plane of polarization and the propagation direction of the light beam is indicated by the broad arrow. If the crystal is oriented such as to give A_{max} of the pyridoxal form at 355 nm, the plane of polarization nearly coincides with the plane of projection of the molecular dyad.

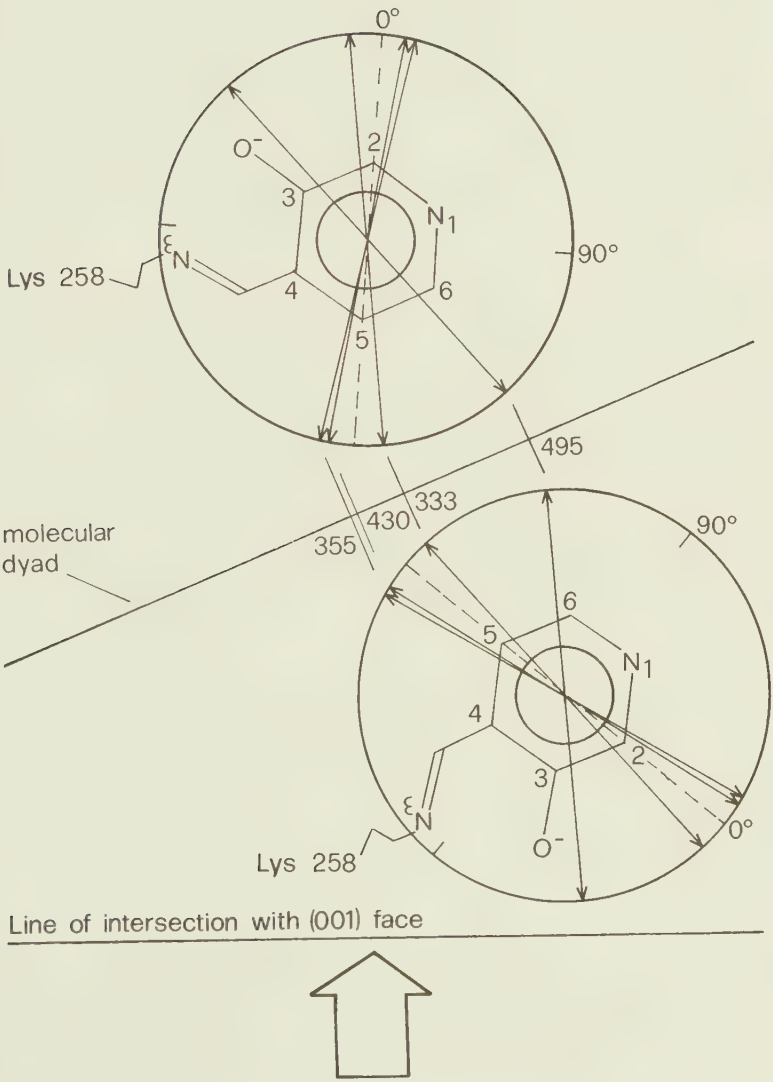

0°
O⁻
2
3
N1
ε
N
Lys 258
4
6
5
90°
495
333
430
355
molecular
dyad
90°
6
5
N1
4
2
3
ε
N
O⁻
0°
Lys 258
Line of intersection with (001) face

Fig. 18. Projection of the transition dipole moment of the two coenzyme rings onto the plane containing the molecular dyad perpendicular to the (001) face (plane of projection of the molecular dyad on (001) face). This plane lies in the plane of the paper. If the crystal is oriented such as to give A_{max} of the pyridoxal form at 355 nm, the plane almost corresponds with the plane of polarization. The propagation direction of light is indicated by the broad arrow that is perpendicular to the (001) face of the crystal. The orientations of the transition dipole moments (⟷, accuracy 10 to 30°) of the coenzyme rings in the different transamination intermediates (indicated by their λ_{max} values) are taken from Song (personal communication to D. Metzler and P. Christen).

crystals with main faces $(0\bar{1}1)$ and $(01\bar{1})$ were used. The results obtained to date are summarized in Table V. A quantitative treatment of these data aiming at the determination of the spatial orientation of the two coenzyme rings in the different functional states is in progress (G. Eichele, J.N. Jansonius, H. Kirsten, and P. Christen, in preparation).

Taking into consideration only the angle δ (Fig. 19A) the possible conclusions regarding a reorientation of the coenzyme ring in the different transamination intermediates are limited. In the case of the pyridoxamine form (λ_{max} 333 nm), it is not possible to draw any conclusion regarding the orientation of the coenzyme. The transition dipole moments of the coenzyme chromophores appear to interact in the pyridoxal and the pyridoxamine form with similarly oriented plane waves. In contrast, in the semiquinonoid intermediate (λ_{max} 495 nm) the large angle δ (Fig. 19A) indicates that the coenzyme rings have undergone a rotatory movement. If the rings were similarly oriented as in the pyridoxal form, their transition dipole moments would interact with a plane wave that is similarly oriented and the orientation for maximum absorption would not have changed much. The large angle δ indicates, however, that they project onto the second plane wave, orthogonal to the first, which is only possible if the coenzyme rings have rotated considerably out of the original plane.

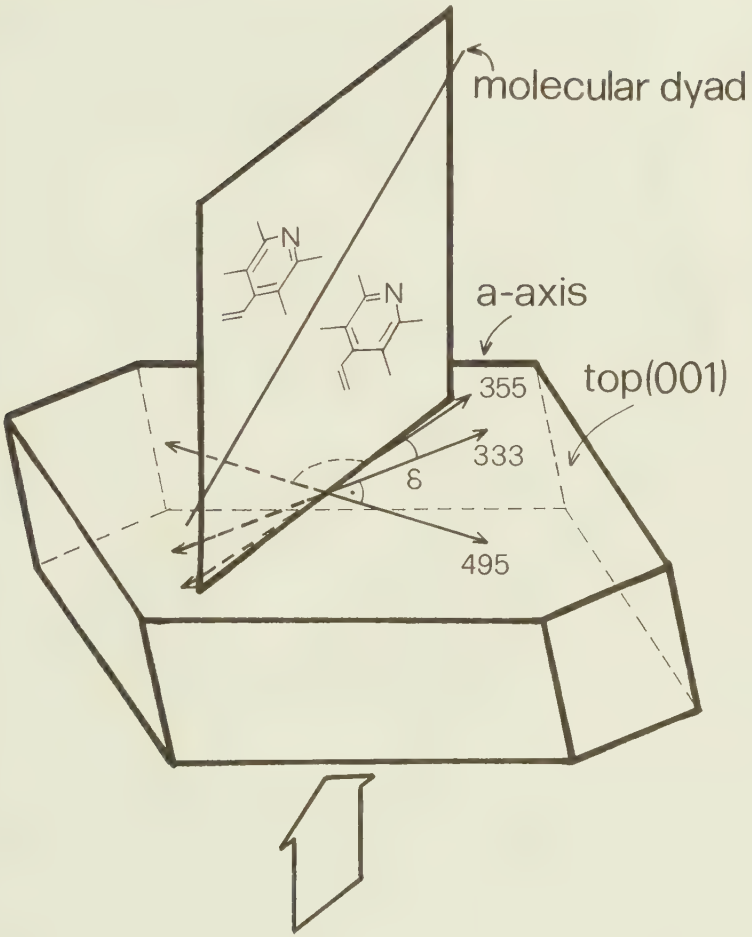


Fig. 19A. Orientation of the crystal corresponding to maximum absorption. As in Figs. 17 and 18, the projections of the coenzyme rings in the pyridoxal form onto the projection plane of the molecular dyad are depicted. The arrow indicates the direction of propagation of the light beam. The orientations of the plane of polarization (⟷) giving A_{max} in the different functional states at their characteristic wavelengths are indicated.

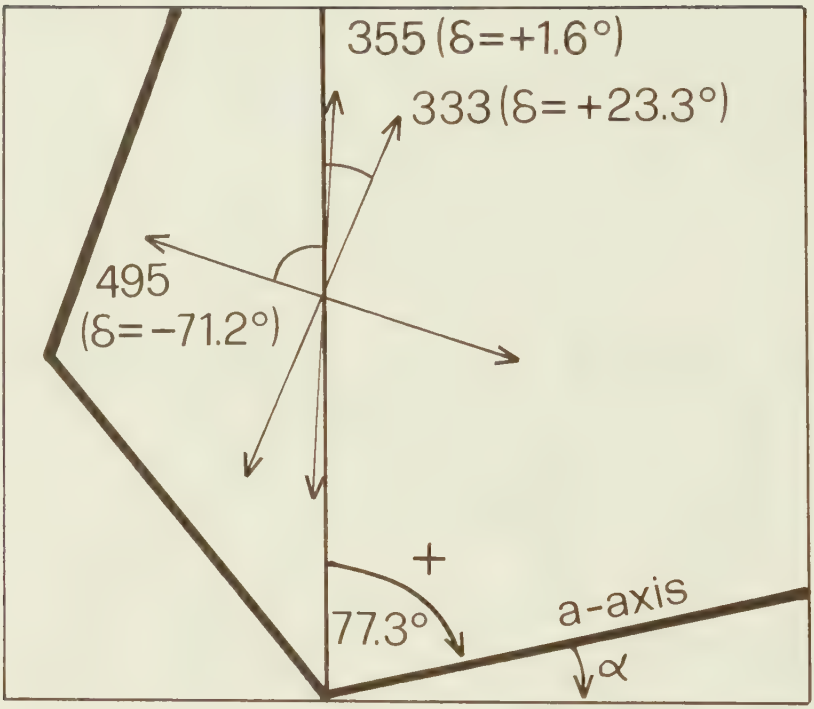


Fig. 19B. Determination of the angle δ between the direction for A_{max} and the projection of the molecular dyad onto the (001) face. The (001) face of the crystal lies in the plane of the paper. The projection of the molecular dyad forms an angle of 77.3° with the a-axis (Eichele et al., 1979). The orientation angle α between the a-axis and the vertical to the plane of polarization was determined experimentally (see Fig. 16 and Table IV). The sign of the angle α is taken positive towards the a-axis: positive sign corresponds to an anti-clockwise revolution of the crystal viewed from above in our experimental set-up, *e.g.* against the direction of propagation of the light.

The following relationship holds: $\delta = \alpha + 77.3° - 90°$.
For the different functional states:

Pyridoxal form	$\delta = 14.3° + 77.3° - 90° = 1.6°$
Pyridoxamine form	$\delta = 36° + 77.3° - 90° = 23.3°$
Semiquinonoid intermediate	$\delta = (-58.5°) + 77.3° - 90° = 71.2°$

TABLE V. Linear dichroism data of transamination intermediates of AspAT in triclinic crystals (space group P1) positioned such that the light beam was perpendicular to the faces $(01\bar{1})$ and $(0\bar{1}1)$[a]

Transamination intermediates	λ_{max} (nm)	orientation angle $\alpha \pm$ S.D.	polarization ratio = A_{max}/A_{min}	angle δ [b]
Pyridoxal form				
at pH 7.5	355	- 12.7 ± 2.3° (N=13)	4.6 ± 0.9 (N=8)	- 62.2°
at pH 5.1	430	- 7.3 ± 5° (N=3)		
	355	- 13.2 ± 2.6° (N=3)		
Semiquinonoid intermediate	495	87.5 ± 3.5° (N=9)	4.9 ± 0.4 (N=4)	- 73.3°
	333	- 13 ± 3.6° (N=3)		
	355(sh)	A_{max} uncertain		
Pyridoxamine form	333	- 23.8 ± 8° (N=5)	1.5 ± 0.15 (N=5)	+ 38°

[a] The data were collected under the same conditions as given in Fig. 16 and Table IV, except for the different mounting of the crystals.

[b] The angles δ were calculated according to the procedure described in Fig. 19B. The molecular dyad and the transition dipole moments are projected onto the $(0\bar{1}1)$ face of the crystal and viewed against the propagation direction of the plane-polarized light. The angle between the trace of the molecular dyad and the a-axis is 40.5°.

4. Concluding Remarks

High resolution (2.8 Å) X-ray crystallographic analysis of the position of the coenzyme ring in different functional states (Eichele, 1980; Ford et al., 1980; J.N. Jansonius, personal communication) has shown that the reorientation of the coenzyme corresponds with a mainly rotary movement around an axis in the region of the N1 atom more or less parallel to a line connecting C2 and the middle of the C5-C6 bond. The phosphate group and the N1 atom retain their positions. The reorientation of the ring is similar to that proposed by Ivanov and Karpeisky (1969) who suggested that the ring rotates around an axis passing through C2 and C5. The occurrence of reorientations of the coenzyme ring is in agreement with linear dichroism data of Metzler et al. (1978) and Makarov et al. (1981). Additional difficulties in the interpretation of the linear dichroism data of triclinic crystals of AspAT may arise from the lattice-induced functional asymmetry of the two subunits of the enzyme dimer in the crystal. X-ray crystallographic study of the N-(5'-phosphopyridoxyl)-L-aspartate apoenzyme complex has shown that extensive conformational changes take place in only one of the two subunits (Eichele et al., 1979). A lattice-induced functional asymmetry of the two subunits has been detected in kinetic studies with suspensions of microcrystals (*Section II*, and Kirsten et al., 1982). It is uncertain, if the observed functional asymmetry also involves different changes in orientation of the coenzymes of the two subunits.

In conclusion, a quantitative interpretation of the linear dichroism data available to date is not possible. However, the results unequivocally indicate that the formation of the semiquinonoid intermediate with the substrate analog erythro-3-hydroxyaspartate is accompanied by a rotatory movement of the coenzyme.

IV GENERAL CONCLUSIONS

Substantial differences between the kinetic characteristics of the crystalline enzyme and of the enzyme in solution as they were found in the case of AspAT have also been reported for several other enzymes. The main problem of interpretation is the differentiation between different possible causes of the change in activity, *e.g.* between crystal packing effects on the one hand and effects from crosslinking of the crystals or from components of the crystallization medium on the other. The present study avoids experimental artefacts, the decrease in activity can be unambiguously attributed to lattice effects. There are several ways conceivable how lattice effects cause differences in the kinetic properties of a crystalline enzyme. First, the active site geometry can be distorted. The comparison of the spectroscopic characteristics of the crystalline enzyme with those of the enzyme in solution provide information about the overall conformation of the active site. In the case of crystalline AspAT the pK_a' value of pyridoxal-5'-phosphate is not significantly shifted relative to that of the enzyme in solution. The overall consistency of the spectral properties indicates a conserved overall conformation of the crystalline enzyme. However, a limited distortion of the conformation of the active site is not ruled out. Secondly, intermolecular contacts can include the intrusion of elements of neighboring molecules into regions of catalytic importance. Hindered access of the substrate to the active site can thus reduce the activity of the crystalline enzyme. This possibility is ruled out in the crystals of AspAT. X-ray crystallographic as well as kinetic analyses have shown that the active sites are accessible to even large molecules such as the coenzyme-substrate analog N-(5'-phosphopyridoxyl)-L-aspartate. Third, the enzymic transamination reaction catalyzed by AspAT is accompanied by conformational rearrangements of the enzyme moiety which are integral

features of the catalytic mechanism of action. In the crystal, the conformational flexibility may be constrained by lattice forces that overwhelm the forces inducing the conformational changes. In crystals of AspAT the reduced catalytic efficiency can indeed be attributed to a reduced conformational flexibility. In the crystal, the two subunits of the enzyme dimer are functionally nonequivalent. X-ray crystallographic analysis of the apoenzyme complex with N-(5'-phosphopyridoxyl)-L-aspartate has shown that only one of the two subunits undergoes a conformational rearrangement. The kinetic analyses, *e.g.* the determination of the Michaelis-Menten parameters and the irreversible mechanism-based modification of either the high affinity (slow) or the low affinity (fast) subunit, have clearly shown that the two subunits are functionally nonequivalent. The functional asymmetry of the crystalline enzyme dimer is due to different sets of lattice contacts of the two subunits in the triclinic crystals.

The determination of the functional properties of a crystalline enzyme not only aims at a comparison of the enzyme in solution and in the crystal but also assesses the dynamics of the enzyme. It thus assists in defining a dynamic mechanism of the enzyme, particularly, it contributes to the understanding of the importance of ligand-induced and syncatalytic conformational adaptations.

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APPENDIX

TABLE III. Orientation angles α of transamination intermediates or adsorption complexes of AspAT in triclinic crystal (space group P1) [a] placed onto the main faces (001) or (00$\bar{1}$).

Additions	λ_{max} (nm)	orientation angle α
Pyridoxal enzyme		
+ DL-2-methylaspartate (35 mM)		
at pH 7.5	430	25° (N=2)
	355	11/15°
at pH 5.1 [b]	430	32° (N=1)
	355	
+ 2-oxoglutarate (200 mM)	430	20° (N=2)
	355	10°
+ oxalacetate (50 mM)	355	45° (N=1)
+ N-hydroxyglutamate (10 mM)	375	~ 85° (N=1)
+ aspartate (4 mM) / oxalacetate (0.2 mM)	365	10° (N=2)
	333	22°
+ cyanoborohydride (5 mM) [c]	340	30° (N=1)
+ phenylhydrazine (20 mM) [d]	430	70° (N=2) [e]
Pyridoxamine enzyme		
+ aspartate (200 mM)	333	34° (N=1)
Complexes of apoenzyme with analogs of covalent coenzyme-substrate intermediates		
+ N-(5'-phosphopyridoxyl)-L-aspartate (1 mM) [f]	333	29° (N=3) [g]
+ N-(5'-phosphopyridoxyl)-L-alanine (2 mM) [f]	340	21° (N=3) [h]
+ N-(5'-phosphopyridoxyl)-3-iodo-L-tyrosine (1 mM) [f]	333	43/35° (N=2) [i]

[a] For conditions see *"Formation of enzyme-substrate intermediates"*. All data shown were obtained with a UMSP I microspectrophotometer. No polarization ratios are given, because this instrument is not constructed for measurements in polarized light; the photomultiplier response is not isotropic.

[b] In 20% (w/v) PEG, 50 mM sodium acetate, pH 5.1.

[c] Reduction performed at pH 5.1, 1 h.

[d] The solution of phenylhydrazine was freshly prepared before use (15 min).

[e] Crystals examined with respect to the $(0\bar{1}1)$ face, angle $\alpha = 58°$.

[f] Synthesized according to Severin et al. (1969) with modifications described by Eichele et al. (1979).

[g] Crystals soaked for 1 day at 4°C. The apoenzyme crystals disintegrate when in the solution for soaking 50 mM sodium phosphate is replaced by 20 mM Hepes, pH 7.5.

[h] Crystals soaked for 5 h at room temperature.

[i] Crystals soaked for 4 days at room temperature.

CURRICULUM VITAE

Heinz Kirsten, born November 8, 1950 in Zurich, citizen of Hasle bei Burgdorf/BE.

Education:

1966 to 1971	Kantonsschule Appenzell Ausserrhoden, Trogen, Matura, Typ C.
1971 to 1978	Swiss Federal Institute of Technology, Zurich, Dept. of Natural Sciences; Diploma 1977, Dipl. Natw. ETH. Diploma of mastership in chemistry, 1978.
1977 to 1982	University of Zurich, Institute of Biochemistry, Assistent, Thesis under guidance of Professor P. Christen.
since 10/1981	Kantonsschule Hohe Promenade, Zurich, Employment for teaching chemistry.

List of lecturers whose lectures, exercices and practical courses I attended:

General Botany	Hess, H., Kern, H., Matile, Ph., Moor, H., Ruch, F.
Biochemistry	Carafoli, E., Martius, C., Semenza, G.
General and Molecular Biology	Bättig, K., Dutler, H., Rudinger, J., Schwyzer, R., Turner, D.
Cell Biology	Eppenberger, H.M., Koller, T., Lezzi, M.

Chemistry	Arigoni, D., Clerc, J.T., Eschenmoser, A., Fringeli, U., Geier, G., Jeger, O., Meier, W.M., Müller, K., Pretsch, E., Schneider, W., Seibl, J., Simon, W., Vasella, A.
Genetics	Hütter, R., Ulrich, H., Würgler, F.
Mathematics	Bühlmann, H., Meier, K.
Microbiology	Ettlinger, L., Fiechter, A., Hütter, R., Leisinger, T.
Physics	Busch, G., Kneubühl, F., Wachter, P.
Teaching and Pedagogics	Christen, H.R., Fischer, H., Huber, G.
Zoology	Camenzind, R., Ulrich, H., Ziswiler, V.